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Synthesis of an Oral Biopolymer Platform for the Delivery of Paclitaxel

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by

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A dissertation submitted to the

Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Pharmacy

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Johannesburg, 2022

DECLARATION

I, Vanessa Tendai Chivere, declare that this dissertation is my own work. It has been submitted for the degree of Master of Pharmacy in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.



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Signed on the 13th day of ……July….. 2022

RESEARCH OUTPUTS

Publications

Vanessa T. Chivere, Pierre P. D. Kondiah, Yahya E. Choonara and Viness Pillay. Nanotechnology-Based Biopolymeric Oral Delivery Platforms for Advanced Cancer Treatment. Cancers 2020, 12, 522.

DEDICATION

This work is dedicated to my parents. All that I am today is because of you.

ACKNOLEDGEMENTS

In the name of the Almighty, without faith and guidance, none of this would have been possible.

To my mother, Betty Moyo, for being my pillar of strength in the hardest of times, and my most vivacious cheerleader in the best of times. Thank you for always encouraging me to strive for more, and for always leading by example. To my father, Taswell Chivere, for teaching me the value of hard work and perseverance. Thank-you both for your sacrifices, your unconditional love, and your unwavering support. I will never truly be able to express my gratitude.

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This was not an easy journey. It was filled with ups and downs, tears of both joy and frustration. But it has been worth it; I have learnt that to truly succeed, one cannot fear failure.

Abstract

Chemotherapy remains the primary form of treatment for cancer. Intravenous use of certain chemotherapeutic drugs have been linked to increased risk of morbidity associated with the risk of thrombosis and other debilitating side effects. The oral route of drug administration is the most convenient but has its own share of drawbacks. Low oral bioavailability and drug hydrophobicity, significantly affect the implementation of oral administration. In this research we discuss the synthesis of an oral biopolymeric platform for the delivery of paclitaxel (PTX). Poly (lactide-co-glycolide) (PLGA) and methoxy polyethylene glycol (mPEG) were used for the entrapment of the hydrophobic drug, PTX. The emulsion solvent evaporation method was used to load PTX into the hydrophobic core of the mPEG-PLGA copolymeric nanoparticles. The physiochemical properties of mPEG-PLGA formulation and the PTX loaded formulation were characterised and compared by FTIR, thermogravimetric analysis (TGA) and differential scanning calorimetry analysis (DSC) equipment. The size and morphology of the formulations were confirmed using zeta sizer and SEM, respectively. The mPEG-PLGA nanoparticles showed optimal particle characteristics and exhibited a PTX entrapment efficiency of 78.6 % and a drug loading efficiency of 3.74%. Ex vivo cytotoxicity studies were carried out on the A549 cells, a human lung adenocarcinoma cell line of alveolar epithelial origin. The PTX-loaded mPEG-PLGA nanoparticles showed significantly improved cytotoxicity in A549 cells when compared with PTX only.

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Calibration curve: $y = 0.0448x + 0.0311$ (Equation 3.4)

Cell viability (%) $= \frac{\text{sample} - \text{blank}}{\text{control-blank}} \times 100$ (Equation 3.5)

List of Commonly used Abbreviations

CPNP- Copolymeric nanoparticle

PTX- Paclitaxel

PLGA- Poly(lactic-co-glycolic acid)

mPEG- Methoxy poly(ethylene glycol)

TF- Transferrin

TFR1- Transferrin receptor 1

ATR-FTIR- Attenuated Total Reflectance-Fourier Transform Infrared

NP- nanoparticle

ODP- Oral delivery platform

DSC- Differential Scanning Calorimetry

MW - Molecular Weight

NMR- Nuclear Magnetic Resonance

PDI- Polydispersity Index

SEM- Scanning Electron Microscopy

Tg- Glass Transition

TGA- Thermogravimetric analysis

XRD- X-Ray Diffraction

EDC- 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

NHS- N-Hydroxysuccinimide

DMSO- Dimethyl sulfoxide

DCM- Dichloromethane

FITC- Fluorescein isothiocynate

PBS- Phosphate buffered saline

CHAPTER ONE

Introduction

1.1. Background into oral drug delivery of chemotherapeutics

In 2018, the World Health Organization (WHO) reported an estimated 9.6 million cancer-related deaths, making cancer the second leading cause of death in the world [1]. Lung cancer is the prevalent cancer, with non-small-cell lung cancer (NSCLC) accounting for most lung cancer deaths (> 85 %) and cigarette smoking being the major risk factor [2] [3]. Prognosis has been estimated to be less than 1-year [4]. Lung cancer is classified into adenocarcinoma (most common), large cell carcinoma (10 %) and squamous cell carcinoma (25 %) [5].

To date chemotherapy remains the primary form of treatment for cancer. Oral administration is the most convenient route of delivery, but it remains the most challenging route of delivery for anticancer drugs [6], due to hydrophobicity and low oral bioavailability. Paclitaxel (PTX), an anticancer drug of the taxane class is highly potent as it works against several solid human tumours. PTX has become the standard treatment for ovarian, breast and lung cancer, as single or combination chemotherapy [7]. PTX promotes stable polymerisation of tubulin, locking the tubulin in place, forming dysfunctional microtubules, and resulting in cell death [8].

Intravenous Infusion (IV) of PTX has shown several drawbacks due to the cremophor vehicle used to solubilise PTX. The cremophor EL is toxic (non-specific cytotoxic), causes hypersensitivity reactions, vasodilation, lethargy, laboured breathing, and hypotension. IV use is also linked to the increased risk of morbidity associated with potential thrombosis. Oral administration of PTX is therefore a more convenient route of delivery. Oral administration of PTX is highly advantageous but has its own share of drawbacks mainly that PTX has poor water solubility (<0.03mg/ml) due to its lack of ionisable groups, and low oral bioavailability (<10 %) [8]. PTX undergoes extensive P-450 (CYP2C8 and CYP3A4)

mediated hepatic metabolism and 89 – 98 % of PTX binds rapidly to plasma proteins (highly bound to efflux Protein P-glycoprotein, P-gp), hence the low oral bioavailability [9]. Taking the above into consideration, there is a need for the development of oral PTX formulations, with significant bioavailability.

The main focus of this study was to synthesise a mPEG-PLGA copolymeric nanoparticle (CPNP) platform, for the oral delivery of paclitaxel, for the treatment of lung cancer. The CPNPs were utilized to increase the solubility and bioavailability of PTX, and to enhance target site delivery. A Box-Behnken design was completed for acquiring the optimized formulations, with responses most desired for the polymeric systems. The CPNPs were developed through electrostatic interactions using poly(lactic-co-glycolic acid) (PLGA) crosslinked with methoxy poly(ethylene glycol) (mPEG) and loaded with, the drug, PTX, and conjugated with a ligand, Transferrin (TF). Efficacious drug delivery can be hindered by lack of specific drug targeting, hence incorporation of TF [10]. TF is an iron source to generic cells, and it has a high binding affinity to the transferrin receptor 1 (TFR1). TFR1 is significantly expressed in cancer cells, thus TF – iron is highly attracted to cancer cells as compared to normal cells. TF will thus increase drug accumulation at the tumour sites and enhance efficacy of treatment [11].

1.2. Rationale and Motivation

The current intravenous PTX formulation is a mixture containing cremophor EL (CrEL) and ethanol. This currently must be diluted 5 – 20 times in 5 % dextrose or normal saline, before infusion, to avoid plasma precipitation [12]. Intravenous PTX has various pharmacokinetic and physical drawbacks. PTX absorption is limited by the high content of CrEL in the IV formulation, the CrEL forms micelles that entrap the PTX [12], this along with the extensive first pass metabolism of PTX, affects PTX bioavailability (about 30%). The CrEL is cardiotoxic, nephrotoxic and neurotoxic, and there is likelihood of leaching of plasticizers, such as diethylhexylpthalate, from the polyvinylchloride infusion bags [13]. Patient

compliance is also affected due to intravenous infusion being painful and inconvenient. An oral delivery platform is advantageous over IV delivery since it leads to low administration costs (hospitalisation, infusion equipment and nursing assistance) and improved patient compliance hence increased treatment success.

This study was focused on the synthesis of a CPNP platform for oral delivery of PTX, for the treatment of lung cancer. The CPNP platform was thus aimed at elevating solubility and bioavailability of PTX delivery. Target delivery of PTX will reduce cytotoxicity in normal cells, hence increase therapeutic outcome. The small size (50 – 300 nm) of the CPNPs will allow them to penetrate the capillaries, for distribution, and be effectively taken up at the targeted cells [14]. The CPNPs will form a hydrophobic core which will be a reservoir for PTX and hence increase PTX solubility [15].

PLGA, mPEG, and TF were used to make the CPNPs. Because it targets and binds to the transferrin receptor 1 (TFR1), which has been found to be highly expressed in lung cancer cells [16], TF enhances CPNP cell targeting. TF bound ferric iron binds to TFR1 with a high affinity [17]. Iron is taken up in the upper section of the intestine, located near to the stomach. In iron shortage, mucosal transferrin is responsible for increased absorption. Exposure to the stomach's low pH helps keep iron in solution. Ferric iron is converted to ferrous iron by intestinal cell surface reductases, allowing DMT1, an intestinal iron transporter, to absorb ferrous iron. Ferroportin-absorbed ferrous iron is swiftly oxidised to ferric iron by membrane tethered copper-containing oxidases. The newly formed ferric iron attaches swiftly to the iron free form of TF. Once in the circulatory system, iron is transported in the blood by TF, the TF has a high affinity for two atoms of ferric iron [18]. The uptake of iron by transferrin receptors is mediated via clathrin/receptor-mediated endocytosis. TF binds iron and transports it to cells' endosomal compartments. The dissociation of iron from TF is aided by a combination of the degree of TF binding to the TFR1, low pH and ferric iron reduction to ferrous iron. Thereafter, DMT1 can now transfer ferrous iron from the endosome to the cytosol. TF is relatively resistant to gastrointestinal (GIT) digestion;

therefore, TF receptors are extensively expressed in the human GIT epithelium. The TF receptor is also highly expressed on tumour cells; as a result, TF has been proposed as a medication carrier for specific tumours, with the potential to be used as an oral drug carrier [19].

The internalisation of CPNP-TF-TFR1 is facilitated by clathrin-mediated endocytosis. As demonstrated in Figure 1.1, the synthesised CPNPs' tertiary amine groups break the endosome membrane following cellular uptake, resulting in CPNP dissociation and release of the encapsulated medication. As a result, TF targeted delivery increases the amount of PTX delivered to malignant cells. Targeting tumours improves medication therapeutic efficacy while lowering systemic toxicity [15].

PLGA is biodegradable, nontoxic, water-soluble, and a hygroscopic compound which can form solutions of high viscosity at low concentrations [20]. The PLGA metabolites lactic acid and glycolic acid are simply broken down into carbon dioxide and water, resulting in negligible systemic toxicity [21] [22]. Physiochemical parameters (pH, ionic strength of the environment, and temperature), intrinsic properties of the polymer, location of absorption, and processes of hydrolysis all influence the polymer breakdown process, *in vitro* and *in vivo* [22]. PLGA nanoparticles are reported to be internalized in cells via liquid phase pinocytosis and clathrin-mediated endocytosis. This enhances nanoparticle interactions with vesicular membranes, resulting in localized membrane degradation and nanoparticle escape into the cytosol [23] [24]. To enhance functionality, improve drug delivery capabilities, and thus improve cancer therapy, PLGA can be modified or coupled with other polymers. In this study in order to optimize the nanoparticles long-term systemic circulation, mPEG was coupled to the PLGA. mPEG is a non-ionic, hydrophilic polymer that has demonstrated great biocompatibility. The addition of mPEG to the polymeric platform has shown to minimize nanoparticle interaction with digestive enzymes and boost absorption of the encapsulated drug into lymphatic tissue and the bloodstream [25]. Though the mechanism by which mPEG prolongs circulation time is unknown, it has been suggested that the avoidance of

nanoparticle opsonisation by various serum or plasma proteins (opsonins) is what significantly increases nanoparticle residency in the blood [26].

Solvent evaporation was used to encapsulate the model drug, PTX, into the copolymeric platform (PLGA-mPEG). The oil-in-water (o/w) emulsion approach is ideal for water-insoluble drugs, making it a viable option for encapsulating PTX. The mPEG-PLGA and the PTX were both dissolved in an organic solvent in the o/w approach, and the mixture was then emulsified into an aqueous solution using a surfactant/emulsifying agent such as poly(vinyl alcohol), gelatin, polysorbate-80, and poloxamer-188 [25]. Stir rate, viscosity of the organic and organic phases, and temperature all influenced the size of the drug-loaded formulation.

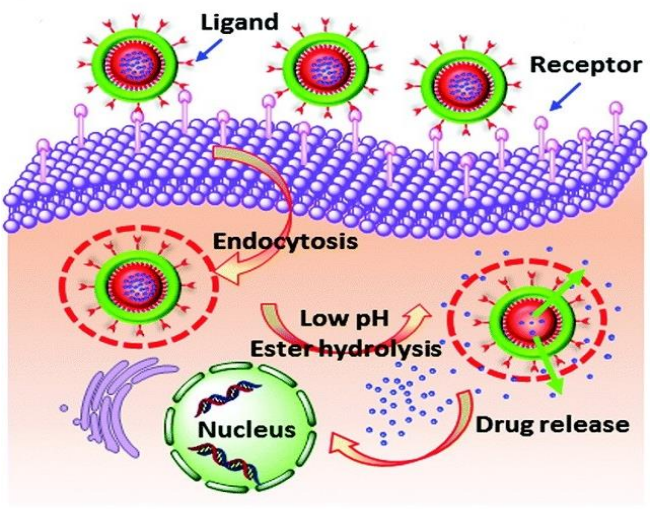


Figure 1.1: Schematic of the ligand cell targeting mechanism and CPNP entry into lung cancer cells. (Ligand - TF) (Modified) (21).

1.3. Possible Therapeutic Applications of the delivery system

This oral mPEG-PLGA copolymeric drug delivery system was analysed in its application for the delivery of a hydrophobic anticancer drug, PTX. The physiochemical characteristics and *in vitro* activity of the delivery system possessed exceptional scientific results, pronouncing the delivery system a suitable oral copolymeric delivery system for PTX. In specific

conditions, such as diabetics, individuals who have previously experienced hypersensitive reactions to conventional preparations, and cases where steroids must be avoided, an oral formulation of paclitaxel is beneficial.

1.4. Novelty of this study

The mPEG-PLGA copolymer was created to provide significant benefits in terms of PTX solubility, bioavailability, long-term administration, toxicity protection, and physical and chemical degradation. Physiochemical studies were undertaken on the developed copolymeric system. The mPEG-PLGA and paclitaxel loaded formulations were evaluated *in vitro.* For accurate drug release evaluation, all *in vitro* drug release studies were conducted utilizing dialysis tubing at three different pH levels. To compare the targeting impact of transferrin conjugated PTX-mPEG-PLGA to PTX-mPEG-PLGA, *in vitro* cellular uptake assays were conducted.

1.5. Aims and Objectives

The aim of this study was to synthesise a copolymeric nanoparticle platform for the oral delivery of paclitaxel, to increase bioavailability and reduce plasma protein binding of PTX. To impart active targeting of the system, a ligand was conjugated on to the formulation.
The following objectives were defined for the study:

1. Selection of appropriate biocompatible, biodegradable, and non-toxic polymers to improve PTX solubility and bioavailability.
2. Development of a crosslinked mPEG-PLGA copolymeric platform loaded with paclitaxel, incorporating crosslinking of polymers to produce a copolymeric delivery system, taking into account the aqueous solubility of the mPEG and PLGA polymers, as well as the properties of the drug for ease of incorporation.
3. The formulation was evaluated on a physicochemical basis, for determination of the stability of the formulation and its characteristic profiles. Scanning Electron Microscopy (SEM), Fourier Transform Infrared (FTIR), Thermogravimetric Analysis

(TGA), particle size, zeta potential, and *in vitro* drug release were some of the characterisation studies undertaken.

4. *In vitro* cytotoxicity analysis employing A549 cell line, determining the biocompatibility of the crosslinked mPEG-PLGA copolymeric nanoparticles.
5. A statistical analysis of *in vitro* results obtained, assessing the statistical significance of the differences between blank mPEG-PLGA formulation and the PTX loaded mPEG-PLGA formulation.

Synopsis of Dissertation

Chapter One entails a comprehensive introduction to the study that covers the background and introduction, and highlights the rationale for the study, thereby also explaining the mechanism of uptake of the delivery system into the cells. It is. A summary of the aims and objectives is included in this chapter, ensuring clarity and analytical sequence throughout the study.

Chapter Two discusses in detail the different biodegradable polymers used in the field of chemotherapeutic drug delivery. This chapter covers the biopolymers used for the development of oral delivery platforms in cancer treatment, specialised oral delivery systems for anti-cancer drugs and the potential challenges of targeted delivery. Overviews of the current route of administration of these chemotherapeutics are essential, therefore indicating a contrast between the newly formulated research and current therapy on hand.

Chapter Three entails the design and optimisation of the mPEG-PLGA copolymeric platform. Using a Box-Behnken experimental design, a series of formulations were synthesized and evaluated. The optimised formulation was synthesised according to the responses from the design formulations. Characterization studies were carried out on the optimised mPEG-PLGA copolymeric platform for determination of chemical, physical, and thermal properties. *In vitro* behaviour of the mPEG-PLGA copolymeric drug delivery system was assessed. Drug loading and release studies were undertaken for determination of the capacity of drug available for delivery. Drug loading and nanoparticle size analysis was carried out on all Box Behnken formulations. *In vitro* cytotoxicity and *in vitro* cellular uptake studies were carried out on human lung adenocarcinoma of alveolar epithelial cells (A549 cell line). This chapter therefore provides a platform for the development, characterisation, and cellular uptake nature of the transferrin conjugated mPEG-PLGA copolymeric system.

Chapter Four summarizes the study done in the form of results concluded and makes recommendations for future works and developments.

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CHAPTER TWO

A review of Nanotechnology-based Biopolymeric Oral Delivery Platforms for Advanced Cancer Treatment

2.1. Introduction

Majority of global deaths are caused by non-communicable diseases, with cancer ranking as the leading cause of death. In 2015 the World Health Organisation estimated that cancer is one of the leading causes of death in 91 of 172 countries [1]. Lung cancer is the most common type of cancer, with the highest number of new cases in 185 countries followed by breast , prostate and colon cancer, respectively [1]. Cancer related deaths are projected to increase rapidly with an estimated number of >20 million by end of 2025 [2].

Current cancer treatment options include surgery, radiation therapy and/or chemotherapy, or a combination of these [3]. Conventional chemotherapy works by interfering with DNA synthesis in rapidly dividing cancer cells, leading to their death or impeded cell replication. Chemotherapy causes numerous side-effects and are known to also affect healthy cells in the body [4]. The inevitable targeting of healthy cells is the major cause of the high mortality rate of cancer patients due to cellular toxicity. Another aggravating factor is the use of high doses of chemotherapeutic agents since the bioavailability of several cancer drugs is relatively poor [4]. The use of high drug doses leads to elevated toxicity in normal cells with the risk of drug resistance developing [4]. Most anti-cancer drugs are administered by intravenous infusion (IV) and in majority of instances requires patients to be hospitalized leading to poor treatment compliance.

It was therefore necessary for researchers to explore the development of oral delivery platforms (ODPs) for cancer drugs to provide self-administered chemotherapy for the treatment of specific types of cancer. The oral route of drug administration is desirable because of its convenience, sustainability, and superior patient compliance. However, numerous cancer drugs are not feasibly delivered via the oral route due to poor aqueous solubility, low oral bioavailability, gastric degradation (stability), the first-pass effect and high affinity binding to biological proteins.

Over the years, research has been undertaken on the use of various biopolymers as a framework for oral drug delivery. Ideally biopolymers should be non-immunogenic and improve the properties of anti-cancer drugs to be delivered via the oral route. At present, several biopolymer-based nanoparticle (NPs) have been developed as chemotherapeutics

but unfortunately majority are for intravenous administration. For this reason, there is still a dire need for developing ODPs for cancer treatment.

The development of effective oral chemotherapeutics could revolutionise cancer therapy, as their use would provide control to the patient. Specialized biopolymers have been synthesized to design ODPs that can survive the harsh GIT conditions for improved anti-cancer drug stability and hence be able to effectively improve drug absorption and bioavailability of anti-cancer drugs [5]. ODPs for chemotherapy have the potential to reduce drug toxicity as doses can be adjusted (via dose banding) to maintain therapeutic concentrations for anti-cancer activity without elevating toxicity to healthy cells [5]. ODPs for chemotherapy have been developed using novel nano-carriers, such as, nanoliposomes, nanoparticles and nano-enabled hydrogels. The use of these nano-carriers ensures the protection of the anti-cancer drug from gastric degradation and boost bioavailability. Furthermore, surface modification of ODPs impart targeted delivery of the anti-cancer drugs [5]. The success of any future ODPs for chemotherapy requires both the use of biopolymer nano-carriers and an appropriate targeting mechanism to ensure effective bioavailability.

This review provides a concise incursion into the use of various biopolymers as ODPs in cancer therapeutics. It also provides an assimilation of the work undertaken in this area and highlights the gaps and challenges faced with the sustainable development of ODPs as a form of chemotherapy for specific cancers.

2.2. Biopolymers used for the development of ODPs in cancer treatment

Biopolymers have become the point of focus for anti-cancer drug delivery due to their biodegradability, biocompatibility and versatility that allows for controlled and targeted delivery for chemotherapeutics. Oral administration of chemotherapeutics is favoured in a myriad of cancer types, but it is limited by several challenges that mostly include drug degradation in the GIT, limited bioavailability, incomplete drug absorption and extensive hepatic metabolism for most anti-cancer drugs. Effective oral drug delivery for anti-cancer drugs is highly dependent on drug stability within the enzymatic and physicochemical GIT environment. As soon as drug reaches the stomach, its stability is affected by the acidic environment and digestive enzymes are continuously secreted along the GIT that can degrade chemotherapeutic drugs before reaching target sites [6]. The unique properties of biopolymers may facilitate oral delivery of chemotherapeutics. For example, the bio-adhesive properties of some biopolymers make them exceptional candidates for promoting drug permeability across the mucosal membrane [6]. In addition, many have inherent properties that enable them to control the release of the incorporated drug using principals of improved solubility, stability and permeability, and reduced toxicity [6]. Table 2.1 provides

a summary of the biopolymers used for the design of nano-enabled ODPs for anti-cancer drugs.

Table 2.1. Summary of Biopolymers, their advantages and possible polymeric complexes.

Biopolymer	Advantages	Polymeric complexes	Drug	Route	Animal model	Tumour type	Therapeutic target	Entrapment Efficacy	Oral vs intravenous drug uptake	References
Gelatin	- Non-toxic - Biodegradable - Inexpensive - Can be cross-linked	-Redox-responsive gelatin nanoparticles -EFGR targeted gelatin nanoparticles	- Gemcitabine - Doxorubicin	- Oral	- SCID beige mice - Nude mice	- pancreatic adenocarcinoma - breast cancer	- Panc-1 cells - MCF-7 cells	- not reported - 82 %	- IC_{50} 17.08 ± 2.32 μM vs IC_{50} value of 8.39 ± 1.79 μM	[9-19]
PLGA	- Biodegradable - Biocompatible	-Magnetic PLGA nanoparticles - PLGA nanoparticles	- Paclitaxel - Cetuximab	- Oral	- Strain mice	- breast cancer - lymphoma tumour	- MCF-7 and U-87 glioma cells - DLS cells	- 82.9 % and 87.3 %	- 64 % vs 18 %	[29-36]
Collagen	- Naturally occurring - Non-antigenic - Biodegradable	- Collagen nanoparticles	- Doxorubicin	- Oral	- *in vitro* study	- liver cancer	- Hep G2 cells	- not reported	- 48 % vs 22 %	[20-22]
PGA	- Hydrophilic - Non-toxic	-PGA nanoparticles	- Cisplatin	- Oral	- Female BALB/c mice	- ovarian	- A2780 cells	- not reported	- IC_{50} 0.14 nM vs	[23-28]

		-PEG-b-(PGA)- b-poly(phenylalanine) nanomicelles	- Doxorubicin			- cancer - lung cancer	- NCI-H460 cells		- IC_{50} 1.5 nM - not reported	
Chitosan	- Biocompatible - Biodegradable - Mucoadhesive - Abundantly available	- Lecithin-chitosan nanoparticles - Chitosan nanoparticles	- Tamoxifen - Doxorubicin	- Oral	- study carried out *ex vivo* - Sprague-Dawley rats	- general tumours	- not reported	- 60 %	- not reported	[38-45]
Alginates	- Biocompatible - Sol-gel transition properties - Mucoadhesive - Non-toxic	-Disulphide cross-linked sodium alginate nanoparticles -pH-responsive alginate nanoparticles	- Paclitaxel - Doxorubicin	- Oral	- Kunming mice	- colon cancer - liver cancer	- HT-29 and CRL 1790 cells - H22 cells	- 77.1 %	- not reported - 1216.7 ng/mL vs 657.7 ng/mL	[46-50]
Hyaluronic acid	- Biocompatible - High viscoelasticity - Non-immunogenic	- Hyaluronic acid nanoparticles	- Doxorubicin - Cisplatin - Paclitaxel	- Oral	- -Female BALB/c mice -	- -ovarian cancer -breast cancer	- A2780 cells - CD44-expressing MDA-MB-231 cells	- 87 ± 5.6 % - 68.76 ± 5.67 % - 28.1 ± 7 %	- not reported	[51-56]
Pullulan	- Hydrophilic - Non-carcinogenic - Non-toxic	- pH-responsive pullulan nanoparticles - Pullulan nanoparticles	- Doxorubicin	- Oral	- nude mice	- breast cancer	- 4T1 cells	- not reported	- not reported	[57-63]

Polymers for ODP formulation include chitosan, gelatin, alginates, PLGA, poly(glutamic acid) (PGA) and hyaluronic acid. They have the capacity to achieve sustained drug release, which is of great interest as it helps overcome tissue barriers until the drug reaches systemic circulation.

2.2.1. Protein-based biopolymers

Protein-based biopolymers are of great interest because they are water soluble, this makes them excellent candidates for use in formulation of ODPs for hydrophobic chemotherapeutics [7]. ODPs using protein-based biopolymers are also essential in cancer therapy as they can enhance drug retention time, drug absorption and can be modified to impart targeted delivery [8]. Enhanced absorption and drug retention time lead to improve chemotherapeutic efficacy, while targeted delivery limits off-target cell toxicity. There are several examples of protein-based biopolymers with specific physicochemical properties for potential use in designing ODPs for anti-cancer drugs.

2.2.1.1. Gelatin

Gelatin is a natural biopolymer with favourable properties for delivery of chemotherapeutics. Gelatin is derived from collagen by acid/alkaline hydrolysis and has been used for the development of several nano-carriers as it is easy to crosslink with other compounds [9] [10]. Gelatin has numerous ionisable groups and has notably resulted in the use of gelatin for colloidal drug delivery systems. Gelatin based platforms can be chemically crosslinked, as shown in Figure 1, by glutaraldehyde, genipin, carbodiimides and N-hydroxysuccinimide; making gelatin an exceptionally adaptable oral drug delivery biopolymer. Gelatin modification yields stealth carriers that can improve drug availability as the drug is shielded from degrading mechanisms, and is able to evade reticuloendothelial uptake, which is important for ODPs.

Drugs can be protected from the acidic gastrointestinal tract conditions and enzymes, thus improving circulation time and accumulation in the leaky vasculature of tumour tissues [11]. Gelatin can also be modified to enhance drug encapsulation efficiency, which in turn, leads to improved bioavailability. Gelatin can be tailor modified to suit the drug being loaded and is therefore a good candidate for oral delivery of hydrophobic chemotherapeutics [58]. Another valuable advantage of gelatin modification is the incorporation of targeting moieties for targeted oral delivery; to spare healthy cells and ensure that after intestinal drug absorption, only the tumour cells are targeted and destroyed [12] [13] 14].

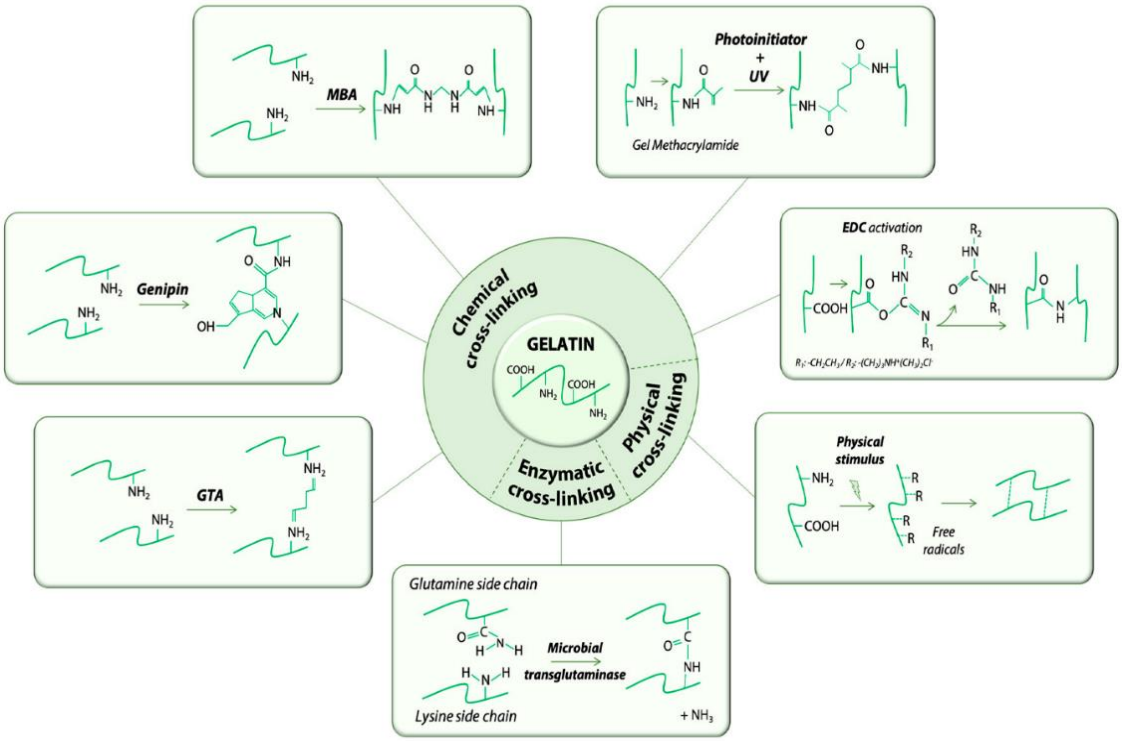


Figure 2.1. Schematic representation of crosslinking methods used to formulate gelatin nano-materials. Adapted and modified with permission from [15].

There has been a significant amount of research done into the use of gelatin for nano-formulations for the delivery of DNA molecules, cancer and tuberculosis agents.

Amit Singh et al. formulated EGFR-targeted redox-responsive gelatin NPs. The gelatin NPs were formulated for targeted delivery of gemcitabine (GEM), for the treatment of pancreatic cancer. The NPs were synthesised by ethanol induced solvation process, to encapsulate GEM. In order to enhance circulation time and enhance target specificity, the gelatin NPs were coated with poly (ethylene glycol) (PEG). The group carried out *in vivo in vitro* studies and the results obtained showed that the encapsulated GEM had a significantly improved cytotoxic profile against the cancer cells and the EFGR-targeted NPs could effectively orally deliver the GEM with improved efficacy as compared to an intravenous solution of GEM in succinimidyl 3-[2-pyridyldithio]-propionate) (SPDP). GEM-SPDP had an IC_{50} value of 8.39 ± 1.79 μM, as compared to the EGFR-targeted redox- responsive GEM loaded NPs, with an IC_{50} value of 17.08 ± 2.32 μM. The GEM-SPDP had a lower IC_{50} value but could not serve as a viable therapeutic option *in vivo* and in clinical use, as it was non-specific and displayed a high binding capacity to any protein in the body [16]. In another study carried out by Sajed Amjadi et al., gelatin was used for the formulation of PEGylated gelatin NPs for the co-delivery of

doxorubicin (DOX) and betanin (BET). The aim was to enhance therapeutic efficacy of the chemotherapy, with reduced side effects.

The formulated NPs were pH-responsive and the loading capacity of DOX and BET was 20.5 % and 16.25 %, respectively. Shape and size of the NPs were analysed, and results showed that the formulated NPs were well rounded, with a uniform size around 162 nm. The pH-response profile was assessed in simulated physiological and tumour environments, and controlled release was shown by adjusting the environmental pH. The formulated NPs were further assessed for cell viability on breast cancer cells (MCF-7), in comparison to free form DOX and BET. The results obtained showed a decrease in cell viability of the MCF-7 cells, hence the group concluded that the developed delivery platform is a promising nanocarrier for cancer treatment [17]. In a recent study, Uyen Vy Vo et al. formulated DOX loaded gelatin-poly (ethylene glycol) methyl ether (mPEG) functionalised porous nanosilica (PNS) NPs. The co-polymeric platform formulated was characterised and results showed that the NPs were spherical in shape, with an average size diameter of 69.60 ± 3,27 nm. The resultant DOX loaded NPs exhibited sustained release of DOX. At pH 7.4, 12.4% of DOX was released from the NPs in the first 2 hours, and later there was a steady increase from 6 hours (22.4%) to 36 h (32.3%). At a pH of 4.5, there was a drastic difference as approximately half of DOX was released from the formulated NPs within 6 hours, and the release constantly increased until 36 hours, at around 70%, thereafter remaining stable up to 96 hours. These results led the group to conclude that DOX/gelatin-mPEG-PNS NPs have a high potential for effective oral delivery of DOX in cancer therapy [18]. Another group carried out a study on the loading efficiency of PNS NPs modified by gelatin, only. The group successfully formulated spherical DOX loaded PNS-gelatin NPs with an average diameter of 70nm. They also assessed the drug loading efficiency (DLE) and drug release of the nanoparticles. The results showed a DLE of 55.90% and a sustained release profile at physiological pH 7.4. Therefore, they concluded that DOX/PNS-gelatin NPs were a promising delivery platform for pH-responsive release of DOX [19].

2.2.1.2. Collagen

Collagen is a naturally occurring protein, abundantly present in animals. It has been used in various drug delivery system applications [20]. Collagen is a biopolymer of interest for use in oral drug delivery, as it is biodegradable, non-antigenic, non-toxic, and biocompatible and has shown synergism with other bioactive compounds. The functional groups in collagen can easily be modified to produce the desired oral drug delivery properties, and the biodegradability of the compound can be controlled by crosslinking. In addition, collagen has

shown properties of being able to resemble the microenvironment of some tumours. This characteristic is beneficial in cancer therapy as it enables the collagen compound to be able to effectively infiltrate the tumour area, to deliver anti-cancer agents [21].

A study was carried out by Zhong Luo et al. where they formulated mesoporous silica NPs end capped with collagen. The aim of the study was to synthesise redox-responsive NPs for targeted drug delivery of anti-cancer drugs. Targeted delivery and cellular uptake studies were carried out on HepaG2 cells. The results obtained showed a 2.2-fold increase in targeted cellular uptake, as compared to the free drugs in intravenous solution. The group therefore concluded that collagen capped mesoporous silica NPs could successfully serve as redox-responsive nanocarriers. The formulated nanoparticles exhibited excellent biocompatibility, cell specific delivery and excellent cellular uptake properties. The collagen capped mesoporous silica NPs can potentially be used for oral chemotherapeutic targeted delivery [22].

2.2.2. Poly amino acid-based and Polyester-based biopolymers

2.2.2.1. Polyglutamic acid (PGA)

Poly glutamic acid (PGA) is a poly amino acid that can be used to form conjugates with anti-cancer drugs. These polymer-drug conjugates have the potential to increase chemotherapeutic drug efficacy and reduce toxicity towards normal cells. PGA is water soluble, therefore is a good candidate for orally delivering hydrophobic anti-cancer drugs. Additionally, PGA is non-toxic to both the environment and humans [23]. PGA is made up of repeating *D*-glutamic acid/*L*-glutamic acid units, and or a combination of both. The molecular weight of the polymer controls the release of the entrapped drug, hence polymers of varied molecular weights are needed, depending on the desired properties and applications of the polymer [24] [25] .

Attention has been drawn to γ-PGA because of its diverse properties, Desale et al. performed a study where they synthesised PEG-*b*-(PGA)-*b*-poly (phenylalanine) micelles for the dual delivery of PTX and an alkylating agent (CDDP). PTX was bound to the poly-phenylalanine core, with 9 % loading capacity while the CDDP was bound to the PGA layer, with 15 % loading capacity. Therapeutic efficacy of the formulated co-delivery platform micelles was tested against A2780 ovarian cancer cells and results obtained showed improved efficacy, as compared to micelles containing PTX or CDDP alone. The group noted that besides multi-drug delivery capability of the PEG-*b*- (PGA)-*b*-poly (phenylalanine) micelles, the micelles also

showed improved stability against disassembly in the gastric region and therefore enhanced tumour delivery [26]. In another study Yi-fei Li et al. evaluated the potential of cisplatin (CIS) loaded PGA-g-methoxy PEG nanoparticles. Analysis tests showed that the drug release rate was accelerated in acidic environment. *In vitro* results proved that the CIS/PGA-g-methoxy PEG NPs could inhibit proliferation of MNNG/Hos osteosarcoma cells. Results from *in vivo* analysis tests carried out in tumour bearing mice, exhibited a comparably higher efficacy with lower toxicity effects as compared to free CIS in intravenous solution. Therefore, it was concluded that the CIS-NPs can be used as a potential osteosarcoma therapy [27].

In 2015, Liadong Deng et al. formulated chitosan-PGA grafted PEG-DOX NPs. Their aim was to engineer drug gastric survivability, improve intestinal permeability, and enhance hemodynamic stability and intracellular activity of DOX. The formulated NPs demonstrated long-circulating properties, improved therapeutic efficacy and lower systemic toxicity as compared to DOX free in intravenous solution at a concentration of 10 μg/mL. The mucoadhesive properties of chitosan permit the formulated NPs to persist in the stomach and adhere to the mucus layer of the small intestine, and subsequently penetrate through the small intestine into the blood circulation. *In vivo* studies of the chitosan-PGA grafted PEG-DOX NPs at a dose of 15 mg/kg, were carried out on Balb/c mice xenografted with Ehrlich ascites tumor, with DOX-HCL (15 mg/kg) intraperitoneal injection (i.p) solution being used as the control formulation. The group treated with the oral NP formulation showed no deaths up to 4 weeks, while the group treated with i.p solution had a survival rate of about 45.5 % over the same period. The chitosan-PGA grafted PEG-DOX NPs exhibited comparable tumour inhibition effects, as that of i.p DOX-HCl, with significantly lower toxicity, enhanced cellular uptake and relative stability in physiological circulation. It was thus concluded that the chitosan-PGA-g-PEG/DOX NPs are a promising nano-platforms for oral delivery of chemotherapeutics [28].

2.2.2.2. PLGA

Poly (lactic acid-co-glycolic acid) (PLGA) is a polyester, copolymer block made up of poly-glycolic acid and poly-lactic acid [29]. It is a biocompatible, biodegradable and non-toxic polymer. Biodegradation of the copolymer means it can easily be removed from the body, therefore, there is less risk of increasing toxicity while delivering anti-cancer drugs. PLGA nanoparticles are a promising platform for cancer therapy, with less side effects and improved drug efficacy [30].

Biodegradation of the copolymer occurs by bulk erosion. Its degradation mechanism allows for potential drug survivability in the acidic gastric environment, as the drug will be

encapsulated at the core. The polymeric chains of PLGA are cleaved by hydrolysis and the monomeric units, lactic acid and glycolic acid, are metabolically excreted from the body [31]. The degradation products are non-toxic to living organisms.

PLGA NPs can be formulated by use of emulsion evaporation, emulsion diffusion, solvent displacement and nanoprecipitation techniques. Figure 2 shows the use of double emulsion-solvent evaporation technique in the formulation of a PLGA based co-delivery platform [32].

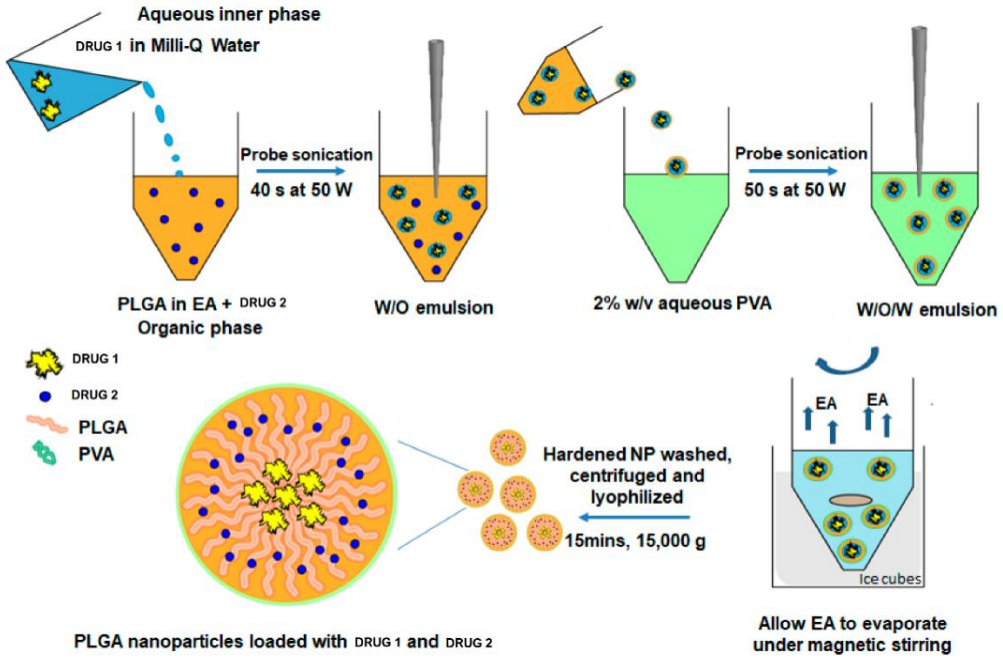


Figure 2.2. Example of a formulation method for the preparation of PLGA NPs. Diagram shows preparation of PLGA NPs for the simultaneous delivery of two different drugs. The drug loaded NPs are prepared by double emulsion-solvent evaporation method. Abbreviations: PVA – poly vinyl alcohol, EA – ethyl acetate. Adapted and modified with permission from [32].

In 2017, Yan-na Cui et al. formulated paclitaxel (PTX) loaded magnetic PLGA (50:50) NPs, surface modified with transferrin (TF). The aim of their research was to investigate the feasibility of the drug loaded magnetic PLGA nanoparticles in enhancing cellular uptake and thus improving the therapeutic efficacy against cancer cells. The drug loaded PLGA NPs were characterised in terms of encapsulation efficiency, size and morphology, magnetic properties, , *in vitro* PTX release, and lastly, cytotoxicity and cellular uptake were evaluated *in vitro* against MCF-7 and U-87 cells. The morphology results obtained showed that the NPs were nearly spherical and approximately 150 ± 20 nm. Encapsulation efficiency (EE) was reported to be 82.9 % and more than 80 % of PTX was released over a period of 10 days, with the release profile following a biphasic profile. The formulated PTX-PLGA NPs showed increased

cytotoxicity against the cancer cells and improved cellular uptake, as compared to the free PTX in solution for injection [33].

In a more recent study, Ajinder Kaushik et al. used PLGA 85:15 to formulate cetuximab (CMB) loaded PLGA NPs [34]. Their aim was to improve drug solubility, drug circulation time and enhance site specificity. The formulated NPs had a molecular size in the 115 – 270 nm range, PDI < 0.50. Biodistribution and pharmacokinetic studies were carried out in healthy strain mice. One group was intravenously injected with free labelled CMB in solution and another group was orally administered labelled formulated NPs. Various organs were harvested from both groups, 24 hours post administration. The results obtained demonstrated statistically significant findings between the two groups. Higher concentrations of CMB loaded NPs were observed in the liver, blood, lungs, bone and brain tissues, as compared to that of the intravenously administered CMB. Oral administration of the CMB loaded NPs may therefore be useful in treatment of tumours in these tissues. Lower distribution of the NPs to the heart and kidneys were reported, thus proving advantageous, in reducing side effects and toxicity. The results from the analytical tests carried out, led this team to conclude that the oral CMB-PLGA NPs showed sustained release and improved drug targeting, as compared to the intravenously administered free CMB [34].

In another study, piperine (PIP) loaded PEG-PLGA NPs were formulated for the targeted delivery of adjuvant breast cancer therapy. Manendra Pachauri et al. reported that the PIP/PEG-PLGA NPs killed MCF-7 cells by apoptotic mechanism. When used in combination with PTX, the PIP loaded NPs exhibited significant reduction in the PTX dose. Results obtained from the study led to the conclusion that the PIP/PEG-PLGA NPs were safe to be used as adjuvant cancer therapy against resistant breast tumours [35]. In 2019, Ankit Saneja et al. carried out a study where they formulated GEM and betulinic acid (BA) loaded PLGA-PEG NPs. Their aim was to improve chemotherapeutic efficacy. The NPs were formulated using double emulsion technique and had a resultant size of <200nm. In vitro cytotoxicity tests were carried out on Panc1 cells and the results showed improved cancer cell toxicity as compared to free drug solution. *In vivo* anticancer activity was assessed on GEM/BA NPs in comparison to GEM/BA injection solution. The results obtained showed that the solid tumour treated with GEM/BA NPs had a mean volume of 195.5 mm^3, while the tumours treated with GEM/BA injection solution had a mean volume of 213.5 mm^3. The group concluded that the GEM/PLGA-PEG/BA NPs could potentially be used to co-deliver chemotherapeutics to enhance antitumor efficacy [36].

2.2.3. Polysaccharide-based biopolymers

Polysaccharide biopolymers consist of repeating simple sugar units joined by glycoside bonds. They are generally inexpensive and abundantly found in nature. They are stable, non-toxic and are excellent candidates for development of biocompatible anti-cancer drug delivery platforms. Biopolymers such as chitosan, alginates and hyaluronic acid have hydrophilic groups that form bio-adhesions with biological tissues, potentially increasing cellular drug retention which is beneficial in oral delivery of chemotherapeutics [37]. Additionally, they can be used to synthesise interpenetrated polymer networks with the desired physicochemical properties. All these properties make polysaccharide-based biopolymers, excellent materials for formulation of “smart” anti-cancer drug delivery platforms that can improve drug solubility, prolong drug release and enhance targeted delivery [37].

2.2.3.1. Chitosan

Chitosan is produced from purified and N-deacetylated chitin. Chitosan production can be optimised to control the final product properties [38]. Chitosan is biocompatible, biodegradable, muco-adhesive and is an efflux pump inhibitor and it enhances drug cell permeation [39]. Its muco-adhesive and efflux inhibitor properties enhance anti-cancer drug cell permeation, increase intracellular concentration and thus help improve chemotherapeutic efficacy. Chitosan has been widely examined as a potential oral absorption enhancer due to its mucoadhesive properties and capacity to open tight junctions between epithelial cells, therefore enabling transportation of macromolecular drugs through “well-organised” epithelia.

The $-NH_2$ and -OH groups of the chitosan can be used as sites for molecule modification. Chitosan modification can either be physical or chemical. Chitosan derivatives offer modified properties for controlled drug release and can impact the pharmacokinetic profiles of the loaded anti-cancer drugs [40]. Chitosan can be used to coat other biopolymers to impart or enhance muco-adhesive properties. Drug release from the chitosan NPs tends to be pH-dependent [41]. Chitosan and its derivatives can be used in the formulation of ODPs for anticancer drugs to increase drug dissolution.

Chitosan has been used to deliver drugs with low water solubility and acid-labile anticancer drugs. For example, tamoxifen (TFN), which is sparingly water soluble and is therefore a good candidate for oral drug delivery. In 2015, S Barbieri et al developed lecithin-chitosan NPs loaded with TFN. The TFN loaded NPs showed muco-adhesive properties and increased drug permeation across the intestinal epithelium. Intestinal permeation of the TFN loaded NPs was assessed in comparison to tamoxifen citrate in ringer solution (160 µg/ml). The amount of TFN

absorbed and transported through the intestinal epithelia, from the tamoxifen citrate suspension, after 4 hours was 0.76 nmol. On the other hand, the amount of TFN absorbed from the TFN loaded NPs was reported to be 1.5 times higher than that of the tamoxifen citrate suspension. When the NPs were used, TFN passed through the intestinal tissue without being transformed by the CYP 450 enzyme, hence more of the drug was able to be found on the receptor site [42]. In another study, Feng et al., also developed a potential ODP for anticancer drugs. They prepared chitosan/carboxymethyl chitosan NPs, loaded with DOX. The DOX—chitosan/carboxymethyl chitosan NPs showed enhanced drug intestinal absorption, throughout the small intestine. Sprague-Dawley rats were used to test bioavailability of orally administered DOX loaded NPs (10 mg/kg), orally administered DOX aqueous solution (10 mg/kg) and intravenously injected DOX solution (2 mg/kg). Blood samples were collected from the tails of rats at predetermined time intervals. No significant DOX was detected in the plasma after oral administration of the free DOX solution, demonstrating poor absorption of the free DOX. The group intravenously treated with DOX solution, resulted in a maximum plasma concentration (582.89 ng/mL) immediately after administration. The c_{max} value of DOX after oral administration of DOX loaded NPs was 208.07 ± 20.17 ng/mL, which was nearly 4-folds, being 2.3-folds higher than that of free DOX solution. The results demonstrated improved intestinal absorption of DOX, when loaded onto the chitosan/carboxymethyl chitosan NPs, and hence improved oral bioavailability. Organs were excised from the rats, 24 hours post administration. The rats treated with oral DOX loaded NPs showed accumulation of drug in the liver (5.87 µg/g tissue), spleen (3.65 µg/g tissue) and lungs (2.58 µg/g tissue). While the rats treated with DOX solution (oral and intravenous), demonstrated DOX concentrated in the kidneys. These results indicated that DOX—chitosan/carboxymethyl chitosan NPs could prolong systemic circulation and the retention time in the mentioned organs, and can therefore be used to target tumours in the liver, spleen and lungs [43].

In 2017, Asad Khan et al., synthesised and characterised carboplatin (CPN) loaded chitosan NPs for the treatment of breast cancer. The CPT loaded NPs had an average size of 277.25 ± 11.37nm and a zeta potential of 31 ± 3.14mV with low polydispersity index. Maximum drug encapsulation was obtained at 58.43%. The CPT chitosan NPs exhibited significant blood compatibility. When analysed *in vitro*, the NPs showed improved cytotoxicity effects against MCF-7 cell line. The group concluded that the CPT chitosan NPs could be used as a potential candidate for cancer treatment [44]. In a more recent study, pH-responsive chitosan-grafted-poly (methacrylic acid)/graphene oxide (CS-g-PMAA/GO) NPs were formulated as a potential chemotherapeutic delivery platform. The NPs were loaded with DOX and the experimental results obtained showed improved biological and physiochemical properties of the drug. At 100 µg/mL, the CS-g-PMAA/GO NPS had comparable therapeutic efficiency, with that of free

DOX in solution for injection. Both treatments demonstrated a tumour survival rate of about 30 %. The CS-g-PMAA/GO NPS had an added advantage of controlled drug release, favourable biodistribution and reduced drug side effects. The CS-g-PMAA/GO NPS demonstrated increased drug release in acidic media. It is well established that the tumour microenvironment has acidic conditions, therefore it was concluded that the NPs may be applied as a potential chemotherapeutic delivery system, compared to free DOX in intravenous solution [45].

2.2.3.2. Alginates

Sodium and potassium alginates have emerged as one of the most extensively explored biomaterials. Their unique physical properties permit for sustained release and targeted delivery of drugs, thus making them favourable BPs for use in oral drug delivery for cancer treatment [46]. This is because of their muco-adhesive properties, biocompatibility, cytocompatibility, sol-gel transition properties, and biodegradation and chemical versatility properties [47]. Alginates chemical versatility properties are beneficial in oral chemotherapeutics delivery, as they can easily be cross-linked and modified to enhance anti-cancer oral drug delivery. Mucoadhesive properties improve anti-cancer therapy absorption in the intestinal wall, therefore improving drug oral bioavailability [47].

Lim Vuanghao et al., published a report on their use of biocompatible disulphide cross-linked sodium alginate derivative NPs, for targeted oral delivery of PTX to treat colon cancer. They formulated self-assembled cysteamine based disulphide cross-linked sodium alginate NPs, aimed at improving the delivery of PTX to the colon cancer cells [48]. The drug loaded NPs were reported to have exhibited 77.1 % EE and a cumulative drug release of 45.1 % in pH 6 medium with GSH. PTX release was based on the breakage of the disulphide linkages in the NPs. The reduction process was catalysed by GSH (reducing agent) in the colonic environment. The GSH is found in abundance in colon cancer cells as compared to normal cells; therefore the condition could help in the high release of PTX from the NPs. At 0.8 µg/mL, the PTX loaded NPs successfully destroyed colon cancer cells, and demonstrated no toxicity towards normal colon cells. On the other hand, at 50 µg/mL; the PTX released from these NPs also destroyed normal cells. Cell internalization into the HT-29 cells was > 70 %. This data therefore prompted them to conclude that the formulated PTX loaded NPs, showed increased therapeutic efficacy as opposed to the free PTX in solution and may be considered as potential ODP for chemotherapeutics targeting colon cancer, with reduced toxic effects.

Another group developed DOX loaded pH sensitive alginate derivative NPs, targeted to the liver. The DOX loaded glycyrrhetinic acid modified alginate-alginate complex (GA-ALG/DOX-ALG) NPs were formulated by self-assembly method. The formulated NPs showed a pH sensitive release profile of DOX, where at pH 7.4, less than 10 % of the doxorubicin had been released, whereas 58.7 % of the drug was released at pH 4. Guo et al carried out an *in vivo* biodistribution study in Kunming mice. DOX biodistribution from the formulated NPs was assessed in comparison to that of intravenous solution DOX-HCL. Significantly different biodistribution in heart, liver, spleen, lung, and kidney tissues was observed in DOX-HCl and GA-ALG/DOX-ALG NP groups, 1 hour after administration. The DOX concentration in the liver of the GA-ALG/DOX-ALG NP group reached 27.6 μg/g, which was significantly higher than that of DOX-HCl (8.1 μg/g). The higher concentration of DOX in the liver was due to glycyrrhetinic acid, having liver targeting properties [49]. Liver targeting led to reduced DOX accumulation in the heart and kidneys, which consequently would lead to reduced cardiotoxicity and nephrotoxicity, as compared to using intravenous DOX-HCL. DOX release from the NPs was still observed 72 hours after administering to mice, while free DOX-HCl was not detectable 24 h post-injection. *In vivo* tumour growth inhibition was conducted and results were obtained by histological analysis of H22 tumour cells, sixteen days after drug administration. The tumor growth inhibition was 78.91% after treatment with GA-ALG/DOX-ALG NPs compared to 51.93% in the DOX-HCl group.

In 2014, Ghislain Garrait et al. developed a novel drug delivery system of chitosan NPs entrapped in alginate microparticles. The aim was to develop an ODP that can protect the encapsulated drug from degradation in the gastric tract. *In vitro* drug release was studied in simulated gastric and intestinal fluids. Regardless of the presence or absence of enzymes, no breakdown of the alginate microparticles was observed. However, in simulated intestinal fluid, with or without pancreatin, the alginate was rapidly disintegrated. The alginate microparticles disappeared within 15 mins, therefore releasing the chitosan NPs for loaded drug release. Experimental results obtained showed pH responsive release of drug, as only 5% of loaded drug was released in the stomach, and at intestinal pH, the drug was completely released. The developed platform proved to be a suitable vehicle to protect anti-cancer drugs from gastric degradation, after oral administration [50].

2.2.3.3. Hyaluronic acid

Hyaluronic acid (HA) is a linear macromolecular mucopolysaccharide, composed of alternating glucuronic acid and N-acetylglucosamine units [51]. The hydroxyl, carboxyl and N-acetyl groups, expressed on the HA make it a good candidate for chemical modification to

create a compound with desired properties, such as sustained drug release and improved drug targeting. HA is biocompatible, biodegradable, has high viscoelasticity, is non-immunogenic and can recognize specific receptors overexpressed in tumour cells [52]. HA receptor CD44 is expressed at low levels in normal epithelial, hematopoietic and neuronal cells. The CD44 receptor is overexpressed in tumour cells, thus making HA a good targeting agent for cancer drug delivery. However, HA is easily degraded hence nitroxide-containing substance, or a hyaluronidase inhibitor must be used in formulation, to protect HA from rapid degradation *in vivo* [53].

The three functional groups on the main chain of HA can be modified, thus, different anticancer drugs can be conjugated to the HA, to formulate HA-drug conjugates. HA-drug conjugates are usually covalently bonded prodrugs and are not easily cracked in the blood but break through enzymolysis or hydrolysis after reaching the target site. HA-drug conjugates can improve drug solubility, therefore is good candidate for use in oral delivery formulations of anti-cancer drugs with low aqueous solubility. HA also imparts change in drug biodistribution and increase *in vivo* half-life, as it leads to increased drug accumulation in the tumour sites due to enhanced osmotic retention and exerts better drug efficacy [54].

In 2015, Cai et al made a novel delivery system, by conjugating cisplatin (CIS) to HA. CIS is widely used for the treatment of most solid tumours, but its use is limited due to the serious side effects. The novel delivery system formulated by Cai et al, led to an increase in the concentration of platinum in the lymphatic vessels, reduced systemic toxicity and side effects, all while inhibiting early tumour metastasis of breast cancer [55]. At pH 7.4, it is expected from the release experiment that most CIS in the NPs will remain in the carrier for a considerable time period. Drug release will occur at the site of endocytosis where the pH is slightly lower, as protonation will trigger release of absorbed drug molecules. Compared to the free CIS in intravenous solution, the HA-CIS conjugate showed increased plasma drug concentration, improved tissue distribution and greatly reduced renal toxicity. *In vitro* drug tissue distribution from the CIS-HA NPs demonstrated 8.31-fold higher distribution as compared to that of free CIS in intravenous solution, after 4 hours of incubation. These results clearly indicate that the drug loaded NPs were internalised by the cells.

In 2019, Nilkamal Pramanik et al. formulated HA-modified graphene oxide (GO) and iron oxide NPs for targeted delivery of PTX and DOX. The resultant NPs exhibited cytotoxicity against breast cancer cell lines, BT-744 and MDA-MB-231. DOX and PTX were successfully loaded and the drug loaded NPs significantly killed CD44-expressing MDA-MB-231 cells but did not kill the BT-474 cells that did not express CD44. Iron oxide was used in the formulation of the

NPs to enable magnetic hyperthermia tumour cell targeting. In conclusion, the functionalized NPs demonstrated improved efficacy in killing tumour cells and thus can be used as a potential platform in cancer therapy [56]. The study was not conducted in comparison to any existing intravenous chemotherapeutic.

2.2.3.4. Pullulan

Pullulan is a hydrophilic biopolymer, it is produced by bi-morphic fungi, *Aureobasidium pullulans*. Pullulan is non-immunogenic, non-toxic, and non-carcinogenic and it exhibits liver specificity. It is a linear and unbranched polymer, made up of repeating malto-triose units connected by α (1→6) glycosidic bonds. It has essential physiological activity due to the vast number of hydroxyl groups found on the main chain. Its hydrophilic properties make it of great importance in delivering poor water-soluble anti-cancer drugs. [57] [58] [59] [60]. Pullulan is an excellent carrier for anti-cancer drugs targeting the brain, liver, lungs and spleen [61]. Although pullulan already has great potential for application into various medical applications, further chemical modification has proven to be advantageous in increasing its utility in the biomedical field. Substitution of the hydroxyl groups with the desired functional groups can be carried out via various chemical reactions [62].

In 2017, Junhui Sui et al. synthesised pH-responsive pullulan-DOX conjugate NPs encapsulating sorafenib (SNB) (P-Dox/S). The formulated NPs were developed as a synergistic delivery system for the treatment of murine breast carcinoma. Characterisation results showed a drug loading capacity of 65.34 % (w/w). The NPs remained stable in physiological conditions. Cell toxicity of P-Dox/S was analysed in comparison to free DOX-HCL, *in vitro* against 4T1 cells. A stronger DOX signal was demonstrated in the nuclei of cells treated with P-Dox/S, after 0.5 hours of incubation. The cell nucleus of the group treated with P-DOX/S began to fade and apoptosis after 6 hours of incubation, with nucleus almost disappearing after 17 hours. *In vivo* tumour accumulation and biodistribution of the NPs was tested in 4T1 tumour bearing nude mice, in comparison to another group treated with free DOX-HCl. Compared with the free DOX-HCl treatment group, the fluorescent signal accumulation of NPs groups was stronger and lasted longer. This may have been due to liver targeting properties of pullulan which was the major component of the nanoparticle carrier. These results led to the conclusion that co-delivery of the chemotherapeutics outperformed the conventional delivery of single/free drugs. Combination therapy has vast potential for use in the treatment of tumours exhibiting drug resistance properties [63].

2.2.4. Biologics as ODPs for anti-cancer drugs

Biological drug delivery platforms composed of living systems are a promising approach to deliver therapeutics. Biological systems have great potential to be used for the delivery of chemotherapeutics as they naturally operate with the central goal of controlled drug delivery and targeted delivery. Biologics such as RNA, DNA, exosomes, erythrocytes and leucocytes can be used as ODPs, as the anti-cancer drugs can be conjugated or encapsulated into the biologic platform [64]. However, oral delivery of biologics is challenging because the biological systems are readily degraded by proteases, nucleases and other enzymes in the gastric tract. Hence, to overcome this the biologic platforms are usually conjugated with other biopolymers.

In this section the use of RNA/DNA delivery platforms will be discussed. We briefly look into their current use as oral delivery platforms for chemotherapeutics and promising future prospective.

2.2.5 RNA/DNA platforms for chemotherapeutic delivery

Research to date encompasses the conjugation of anticancer drugs with small interfering RNAs (siRNA), such as, PGP , XLAP, VEGF, BCL2 and MRP-1. siRNA have shown great potential for overcoming drug resistant genes, as well as improving sensitivity of the resistant tumours to chemotherapeutics. It is noted that the siRNA must be protected from RNAse degradation and there is a need to prevent off-target side effects, hence the use of nanotechnology-based delivery [65]. siRNA refers to a group of synthesized duplex RNA, approximately 20-24 nucleotides long, that can be recognized by the enzyme DICER, thus resulting in gene specific cleavage by complementarily pairing with mRNA [65] [66].

2.3. Nano-Enabled Biopolymer Systems for Specialised Oral Delivery of Anticancer Drugs

In addition to the numerous engineered NP formulation platforms, nano-enabled systems are also used to develop chemotherapeutic delivery formulations. Liposomes, dendrimers, micelles, hydrogels, and metallic and carbon-based carriers can be used to increase chemotherapeutic efficacy, control drug release and improve cell targeting thus reducing cytotoxicity to normal cells [67]. Each carrier system has its own unique strengths. The following section concisely discusses each nano-enabled biopolymer system, chemical structure and use in chemotherapeutic delivery.

2.3.1. Dendrimers

Dendrimers are branched polymeric molecules, which consist of uniform and well-defined shapes and sizes. Dendrimers basic structure consist of three main components, that is, the core, repetitive branching units and terminal groups [68]. The architecture of dendrimers can be controlled, and this makes them attractive molecules for use as drug delivery systems [69] [70]. Hydrophobic anti-cancer drugs can be encapsulated in the dendrimers internal cavity or bound to the dendrimers surface groups through hydrophobic or electrostatic interactions as illustrated in figure 2.3(A), 2.3(B) and 2.3(C).

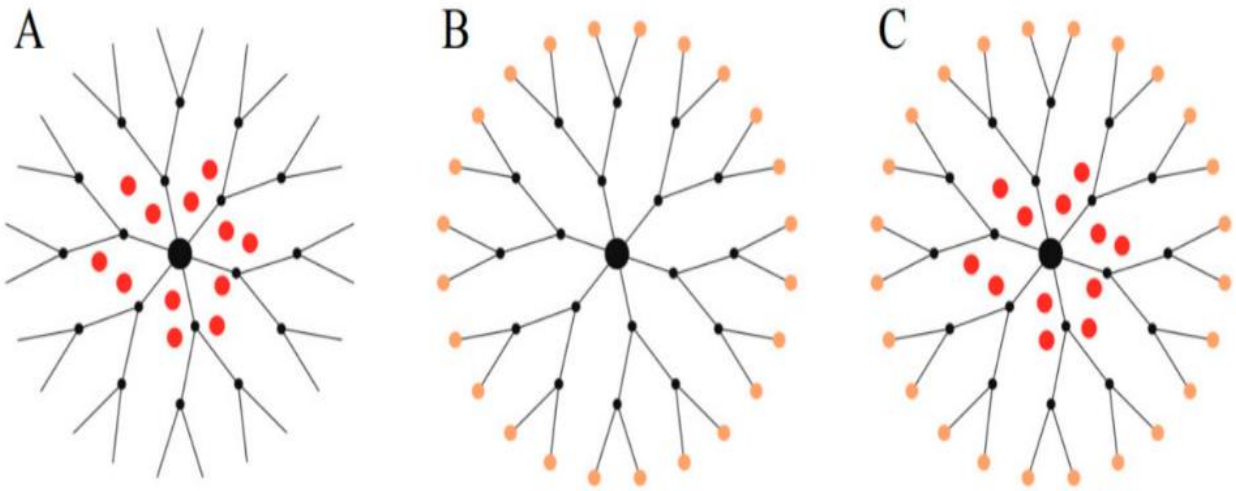


Figure 2.3. Schematic illustration of the three ways of complexation or conjugation of anti-cancer drugs with a dendrimer molecule. A) encapsulation of the chemotherapeutic into the internal cavity, B) surface conjugation of chemotherapeutic onto the peripheral of the dendrimer molecule, C) same dendritic molecule can serve a carrier for both encapsulated and surface-conjugated anti-cancer drugs. Adapted and modified with permission from [71].

There are three types of dendrimers, namely, poly-amidoamine (PAMAM), poly-propylene imine (PPI) and poly-l-lysine (PLL). They all exhibit good biocompatibility, water solubility, flexibility and biodegradability. Dendrimers can be conjugated with other polymers, for example, PEG. Conjugation with other polymers such as PEG is not only used to reduce the cytotoxic effects of dendrimers, but it can also increase plasma circulation time and tumour accumulation, through enhanced permeability and retention effect [72]. Dendrimers can also be modified by conjugation with tumour specific antibodies [73-74]. These modifications are advantageous in cancer treatment as they lead to reduced cell toxicity and improved cancer drug efficacy at lower doses.

The internal cavities of dendrimers, characteristic of their structure, allow for interaction with poorly water-soluble cancer drugs. Sanyamkamdhorn et al. investigated the intramolecular interaction of dendrimers with DOX and TFN [75-76]. Results showed the formation of hydrogen bonds between the drugs and the $-NH_2$ in the PAMAM cavity. The hydrogen bonds, along with electrostatic interactions with the surface amino groups, enabled physical encapsulation of the drugs. Drug encapsulation led to sustained release of the drugs [75].

2.3.2. Nanoliposomes

Liposomes are widely used in drug delivery for systemic administration of drugs. They are microscopic unilamellar or multilamellar particles, made up of membrane like lipid layers [76]. Their ability to encapsulate both hydrophobic and hydrophilic drugs makes them a favourable carrier for anti-cancer drugs. There has been a lot of interest in the use of liposomes as carriers for chemotherapeutics, to increase therapeutic efficiency, reduce toxicity to the normal cells and their small size allows for ease of cellular penetration. Liposomes are naturally occurring phospholipid based amphipathic nanocarriers. When introduced to an aqueous environment, phospholipids self-assemble to form a bilayer with the hydrophobic tails facing each other and the hydrophilic heads facing the aqueous environment. The core formed entraps water and thus water-soluble anti-cancer drugs [77].

Active site targeting can be achieved by grafting the liposomes with monoclonal antibodies (mAbs), antibody fragments, peptides and glycoproteins [78]. Liposomes can be modified to be able to respond to both external and internal stimuli, such as pH changes, light (as shown in figure 2.4A and 2.4B), microwaves, ultrasound and redox reactions [79]. They can also be radiolabelled to be able to determine their biodistribution and to diagnose the tumour. These kinds of liposomes that carry both therapeutic and imaging agents, are called theranostic liposomes [80].

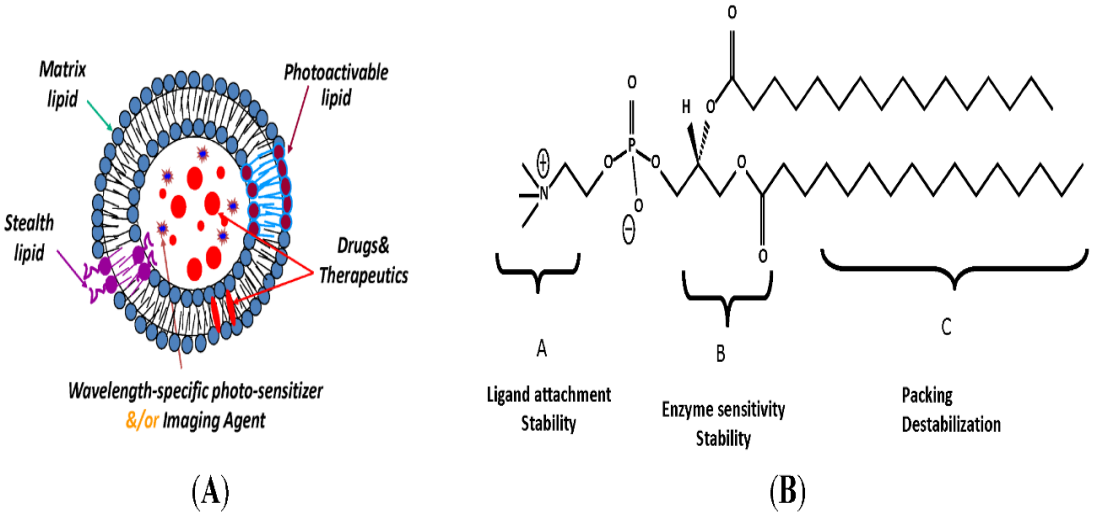


Figure 2.4. A) Photo-sensitive liposomal assembly. Drugs (red), imaging agents and/or second photosensitizer (bright blue), are encapsulate in the liposomes. B) Chemical structure shown as a prototype. The three lipid parts, that is, the head, glycerol backbone and fatty acyl chains, can be modified to generate photo-sensitive materials. Adapted and modified with permission from [81].

Joon Myong Song et al performed an investigation on the liposomal co-delivery-based quantitative evaluation of chemosensitivity enhancement, in breast cancer stem cells [82]. They developed liposomes for the co-delivery of camptothecin (CTN) and glucose-regulated protein 78-siRNA/clusterin-siRNA. The drug-gene conjugate delivery was quantitatively assessed on the cancer stem cells. Compared to the free drug and gene deliveries, the co-delivery system showed that the cancer stem cells had increased transfection efficiency and chemosensitivity. They concluded that their data quantitatively demonstrated that using liposomes for the co-delivery of drugs and genes, offered substantial potential for synergistic anti-cancer therapy.

2.3.3. Nanohydrogels

A hydrogel is a swellable mesh of hydrophilic polymeric chains dispersed in water. Upon swelling, the hydrogels can release drugs previously loaded onto the mesh. Hydrogels have been and are currently being studied for use in oral delivery due to their polymeric chains which are able to closely interact with saliva glycoproteins [83]. The interaction with saliva glycoproteins causes a muco-adhesion phenomenon which leads to prolonged local retention of anti-cancer drugs, this characteristic may prove beneficial in the treatment of oral cancer. Due to the vast need to overcome disadvantages of intravenous administration for most

chemotherapeutics, hydrogels have been investigated for the development of ODPs for drugs such as CIS, PTX, TFN and DOX [83].

2.3.4. Nanomicelles

Micelles are amphiphilic molecules which self-assemble when in contact with a solvent. If the solvent is hydrophilic, the polar parts of the copolymer will be attracted toward the solvent while the hydrophobic components orient away from the solvent. For this to happen, the amphiphilic molecules in solution must exceed critical micelle concentration [84] [85]. A hydrophobic core is formed, and it facilitates the encapsulation of hydrophobic anti-cancer drugs, thus protecting the drugs from the external aqueous environment. On the contrary, when the amphiphilic molecules are exposed to a hydrophobic environment, a reverse micelle is formed.

A micellar system entrapped in a pH-responsive hydrogel was used by Wang L et al. to improve oral bioavailability of docetaxel (DTX). The polymeric micelles were formulated using poly-caprolactone (PCL) and PEG, to allow drug release in the intestinal lumen and subsequently absorption from the small intestine. The *in vivo* studies carried out showed a 10-fold increase in oral bioavailability of DTX and better tumour suppression against the 4T1 breast cancer cells used [86]. In another study, co-polymeric micelles composed of monomethylol PEG- PLGA, d-α-tocopheryl PEG 100 succinate and a stearic acid–grafted chitosan oligosaccharide loaded with DTX were formulated. The aim was to enhance oral bioavailability of DTX. In vitro studies carried showed a 2.52-fold as compared to the free drug suspension [87].

2.3.5. Surface modification for targeted delivery

Nanotechnology delivery systems have gained momentum over the past few years due to their beneficial characteristics such as having a large surface volume ratio, drug protection from degradation, control of drug release hence allowing for the reduction of administered drug doses. As favourable as the delivery systems are, they also have certain limitations. The chemotherapeutic agents carried by the nano-delivery systems are non-cell specific. The unwanted attack on healthy cells is the main reason for the failure of conventional nano-cancer treatment. Hence, nanotechnology delivery systems have had to further advance, to develop novel drug delivery strategies that are able to overcome non-specificity issues, thus the use of targeting moieties as shown in figure 2.5 [88].

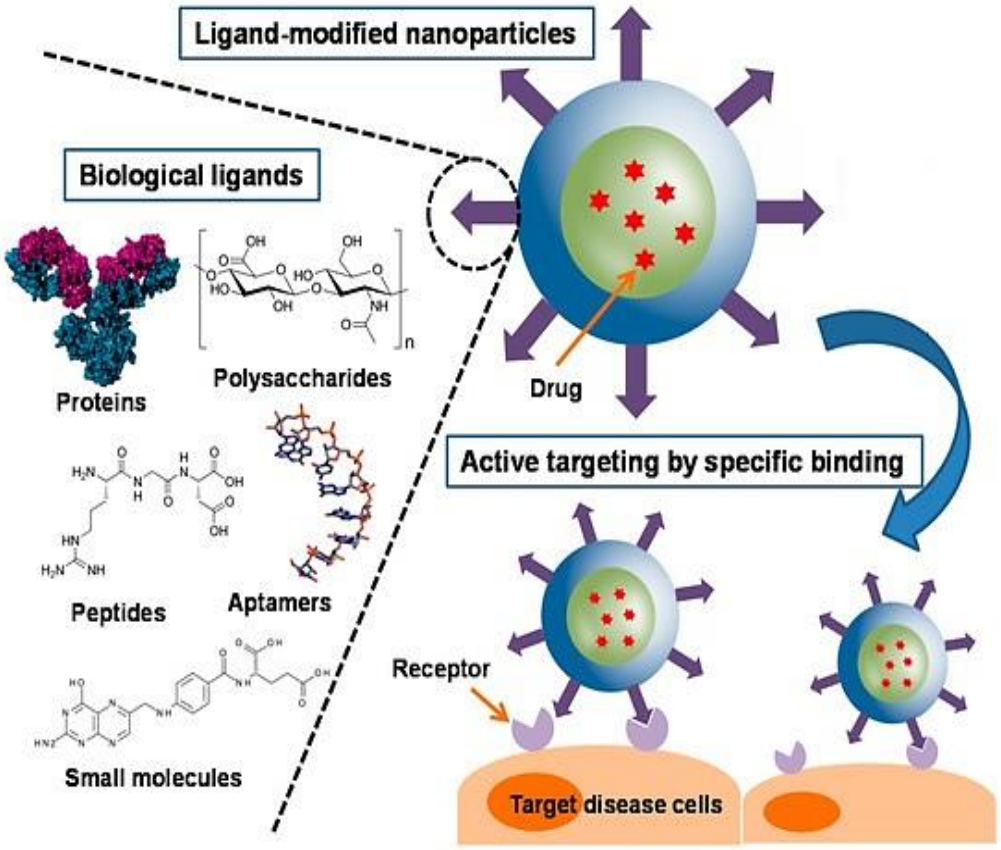


Figure 2.5. Schematic representation of a surface modified nanoparticle, highlighting examples of ligands used in surface modification and attachment to receptor site. Adapted and modified with permission from [89].

Surface modification of the nano-delivery systems allows for active targeted delivery of the therapeutic agent into the tumours. There are several compounds that can be used for surface modification, for example, antibodies and ligands such as TF, folic acid (FA), lactoferrins, lectins and mannose derivatives.

FA the most commonly used compound for drug targeting applications, by conjugating it with anticancer drugs or nanocarriers. In one study, PAMAM dendrimers were amine-terminated and thus functionalised with FA and a fluorescein agent (isothiocyanate). This functionalised system acted as both a drug targeted system and a pH-responsive system, for delivering DOX into tumour cells. The developed complexes displayed effective therapeutic efficacy and a high affinity for FA receptors, and thus were able to target the cancer cells and inhibit growth [90].

2.3.6. Stimuli responsive nanoparticles

Apart from conjugating targeting compounds onto the delivery system, targeted chemotherapeutic delivery can additionally be accomplished by chemically modifying the biopolymer platform to respond to stimuli. Stimuli responsiveness is advantageous in cancer therapy as it ensures anti-cancer drug delivery at target site, hence sparing the healthy cells. Stimuli can be classified as either internal or external, for example, pH and temperature, activity of enzymes, light, irradiation and magnetic fields [91]. Chemical modification of the polymers leads to changes in the nano-carrier structure or chemistry, and thus triggers the release of the encapsulated drug in a biological environment, as shown in figure 2.6. Chemically modified polymers respond to the slightest changes in the environment with notable transitions in the physiochemical properties, such as conformation, polarity, phase structure and chemical composition [91]. Change in physiochemical properties at the target site lead to enhanced diffusion and cellular uptake. Currently, bio-responsive drug delivery offers promising results for the delivery of nanoparticle chemotherapeutics.

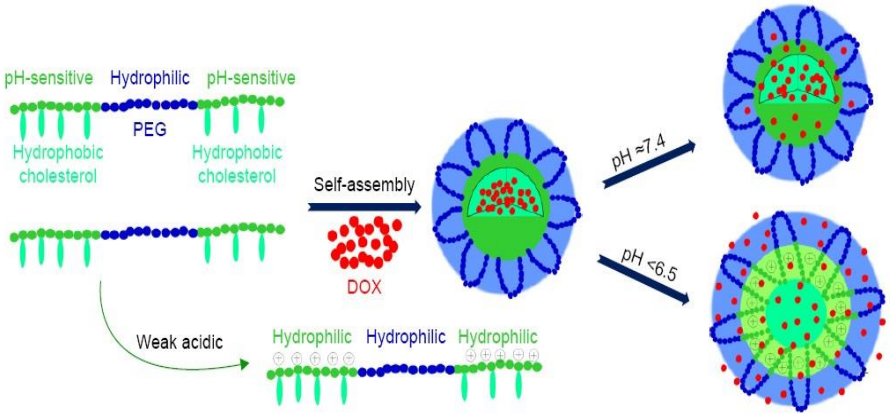


Figure 2.6. Schematic illustration of an example of self-assembled pH-responsive doxorubicin loaded micelles. The micelles were formulated using hydrophilic PEG and hydrophobic pH-sensitive PAE. The micelles will remain stable at normal sites (pH = 7.4), only to respond at the targeted site with the desired pH (pH < 6.5). Abbreviations: PEG – poly(ethylene glycol), PAE – poly(β-amino esters) g-cholesterol, DOX – Doxorubicin. Adapted and modified with permission from [92].

2.4. Challenges with targeted delivery challenges

Tumours show high intra-tumoral heterogeneity, resulting in heterogeneous expression of targets for drug delivery [93]. Targeted therapy is therefore not as simple as originally presumed. Only a subset of the tumour cells expressing the target is initially destroyed, while the tumour cells lacking the target survive and continue proliferating, resulting in a tumour lacking the initial expression target. Drug deliveries targeted at specific tumour cells tend to

leave other tumour cells, most importantly, tumour stem cells. If the tumour stem cells, tumour supportive cells and their supportive environment remain intact it is therefore impossible to completely eradicate the whole tumour and prevent a relapse. Multi-targeting techniques therefore need to be considered in order to achieve complete tumour removal [93].

Another complex issue that arises when it comes to targeted drug delivery is the delivery of the entrapped drugs at their site of action. Current targeting systems simple aim to offload the drug at the site of action, working on the theory that once the delivery system is bound to the target (e.g. membrane receptor) rapid internalisation occurs. Once internalisation has occurred, the drug is expected to be released and subsequently move to its site of action inside the cell, and not diffuse back into circulation [94]. However, the steps to deliver chemotherapeutics are more complex.

Even though targeting the tumour subsequently leads to internalisation at the target site, a wide range of drug delivery systems struggle to offload the drug because of failure to escape from the lysosomal compartments after internalisation [94] [95]. Lysosomal sequestering of drug delivery systems which occurs following internalisation, is the main barrier preventing chemotherapeutics from reaching their target site. For example, less than 1% of administered PEGylated liposomal DOX (intravenous), reaches the target site i.e. the nucleus [96]. The rest of the internalised drug delivery system is found to be entrapped in the lysosomes.

Impaired release of chemotherapeutics may limit treatment efficacy, therefore alternative strategies to tackle these issues are needed.

2.5. Regulation and reality

Several polymer materials have already been approved for use as drug delivery materials by Food and Drug Administration (FDA). However, the regulatory body is having a hard time keeping track with the rapidly increasing development of new polymer-based delivery systems. As of 2015, most of the FDA approved delivery vehicles were either fully liposomal or PEGylated, none of them addressing higher level delivery complexities [97].

With regards to the development and approval of more novel strategies, more data is required from the scientific community to prove efficacy of such formulations. The FDA also needs to play its part with a greater sense of urgency, in order to keep track of these developments. The burden of proof is assumed to mainly rest on academia as well as private and public

sector research facilities, in order to identify the critical factors relating to safety and efficacy of the novel products and strategies [97].

FDA generally classifies liposomes, dendrimers, micelles, nanoparticles and quantum dots as nanocarriers. From these, combination nanocarrier products have vastly gained interest over the years, leading to the development of co-encapsulation of synergistic cancer agents. The majority polymer of the formulations for cancer treatment, in clinical trials or having made their way into the market, are for intravenous use. There are currently several nanomedicines for cancer treatment on the market and in clinical trials. However, to date, a significant number of these formulations are for intravenous use. In addition, despite the FDA full inclusion of combination products on their regulatory evaluations, combination products typically have more hurdles to face before being accepted by the FDA.

The FDA has established regulatory guidance draft documents to provide recommendations for meeting the regulatory requirements. FDA recommends that companies developing novel delivery systems carry out *in vitro in vivo* studies, include details on population to be studied, blood sampling times and analytes to be measured in those blood samples.

2.6. Conclusion and future perspectives

Interest in the application of biopolymers as oral delivery systems for chemotherapeutics, are continuously increasing. This is due to polymers offering favourable characteristics, such as; being biodegradable, biocompatible, non-toxic, hydrophilic and non-immunogenic. The use of biopolymer based ODPs in cancer therapy, allows for the increase in drug solubility, reduction of dose, site specific delivery and protection of the drug from the gastric environment. The drugs can be protected until they reach their site of absorption in the appropriate gastrointestinal segment, for systemic circulation. Oral administration provides prolonged drug exposure, compared to intermittent intravenous infusion, which is an important factor for anti-cancer drugs. A drug with a short half-life can achieve greater systemic exposure by either prolonged infusion or by continuous oral dosing. Patients receiving chemotherapy prefer the oral route over the parental, due to flexibility, convenience and the potential to reduce in-patient healthcare costs. Additional research comparing oral chemotherapeutics and parental chemotherapeutics are thus required as clinical trials. Hence, research further necessitates the use of biopolymers as ODPs for chemotherapeutics, over a longer duration of evaluation. In addition, further studies are required to gather enough data in relation to toxicity of various

polymers. Although many polymers show stability *in vitro* and *ex vivo*, these parameters may still need to be validated *in vivo*, using established diseased models. In the near future, ODPs have the potential to transform chemotherapeutic drug delivery, as they offer solutions to anti-cancer drug solubility issues, drug oral bioavailability and site specificity challenges.

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CHAPTER THREE

Synthesis and Characterisation of Paclitaxel-Loaded mPEG-PLGA Nanoparticles for Oral Delivery

3.1. Introduction

Various anticancer drugs have been used to treat malignancies as diverse as metastatic breast cancer, ovarian cancer in its later stages, and non-small cell lung cancer [1] [2]. Paclitaxel (PTX), one of the anticancer drugs used, promotes stable polymerisation of tubulin, locking the tubulin in place, forming dysfunctional microtubules resulting in cell death. PTX is administered by intravenous infusion (IV), this has shown several drawbacks due to the cremophor EL vehicle that is used to solubilise PTX. Cremophor EL is nephrotoxic, neurotoxic, and cardiotoxic, and there is the likelihood of leaching of plasticisers from the polyvinylchloride infusion bags [3]. Intravenous infusion is painful and inconvenient to most patients, thus affecting patient compliance. An oral delivery platform is therefore preferred over IV administration since it has low administration costs and improved patient compliance leading to increased treatment success [4]. However, drug stability within the enzyme-sensitive and physicochemically harsh gastrointestinal tract (GIT) environment is critical for effective oral drug delivery of anti-cancer drugs such as PTX. [5]. Paclitaxel has poor aqueous solubility (<0.03mg/ml), due to the lack of ionisable groups, and it has a low oral bioavailability of less than 1% due to its extensive P-450 mediated hepatic metabolism. In specific physiological systems, cytochrome P450-dependent metabolic activities and abundance of plasma membrane transporter P-glycoprotein (P-gp), particularly in the colon, kidney, and liver, would eliminate orally administered PTX via first-pass extraction [6] [7] [8]. Taking this into consideration there is a need to develop biopolymeric based drug carriers to enhance PTX oral bioavailability [5].

Several approaches have been used to try and solve the challenges with oral delivery of PTX, including the use of nanomedicines [9]. Nanocarrier-based drug delivery systems have the potential to transform the way drugs are delivered and thus makes them an suitable approach for oral delivery of chemotherapeutic drugs [10]. Prodrugs, dendrimers, micelles, liposomes, and biodegradable polymer nanoparticles are among the most popular and promising pharmaceutical nanotechnology strategies as oral chemotherapy techniques offer controlled, sustained, and targeted drug delivery across a variety of physiological barriers, including the GIT barrier [9] [11] [12] [13].

Polymeric-based platforms provide potential use for effective anticancer drug delivery. These platforms can improve drug solubility, provide effective dosing and sustained drug release

[14]. Poly (D,L-lactide-co-glycolide) (PLGA) is an excellent polyester copolymer, which is biodegradable, biocompatible, and non-toxic [15]. PLGA has been used in application for formulations delivering hydrophobic and hydrophilic drugs thus making it a good candidate for the oral delivery of PTX [16] [17]. To further optimise the delivery system surface modification with methoxy (polyethylene glycol) (mPEG) can be achieved. mPEG enhances the delay of nanomicelle capture, thus allowing longer circulation time in the body and enhanced tumour permeation and retention effect [18] [19].

In this study, mPEG-PLGA was synthesized as the amphiphilic model for the oral delivery of PTX. mPEG and PLGA are FDA approved for clinical use as drug adjuvants [20] [21]. Due to the hydrophobic nature of PTX it was loaded into the hydrophobic core of the copolymeric nanoparticles constituting mPEG-PLGA. The solvent evaporation technique was utilised to synthesise the mPEG-PLGA nanoparticles, loaded with PTX using an emulsion solvent evaporation technique, as shown in Figure 3.1.

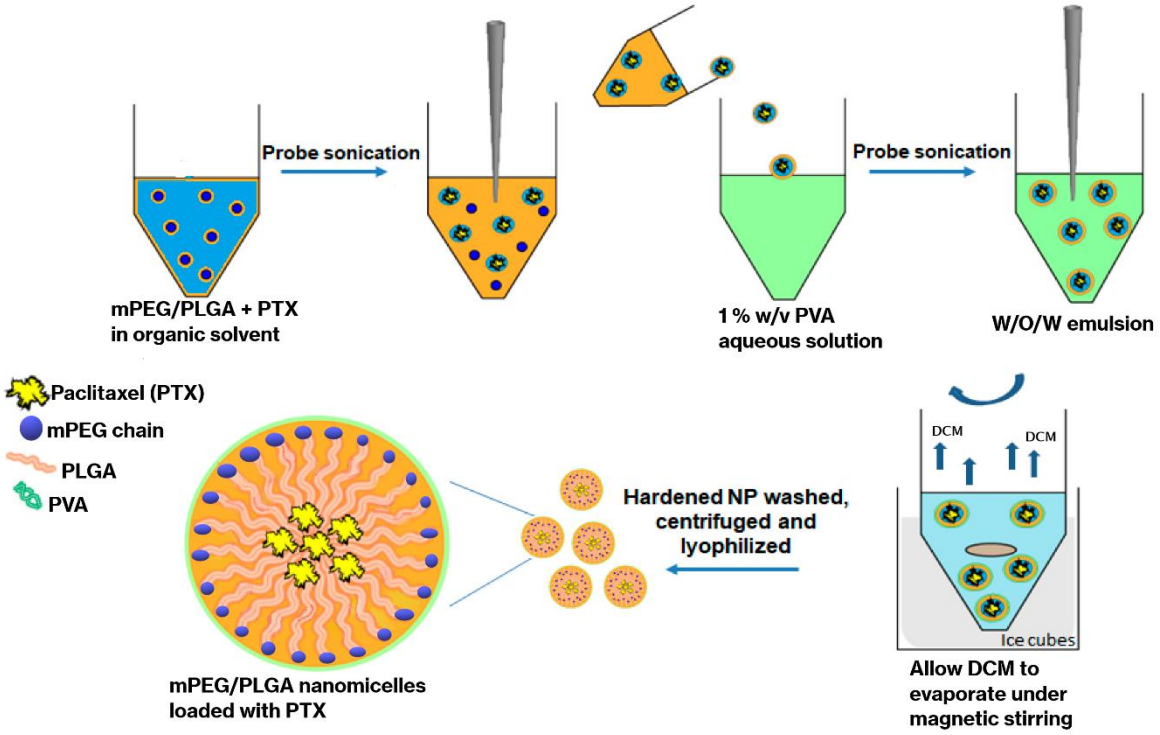


Figure 3.1: Schematic representation of the emulsion solvent evaporation technique used to synthesize PTX-loaded mPEG-PLGA nanoparticles. Dichloromethane (DCM) was used for the organics phase, Polyvinyl alcohol (PVA) was used for the aqueous phase.

3.2.1. Materials and Methods

Poly(D-L-lactide-co-glycolide) (PLGA) (M_W 76 – 114 kDa, 75:25), methyl(polyethylene glycol) (mPEG 750), dialysis tubing (MWCO 3500), paclitaxel (PTX) (model drug), polyvinyl alcohol (PVA) (M_W = 13 – 23 kDa, 87 – 89 % hydrolysed) and PBS buffer tablets (pH = 7.4) were acquired from Sigma-Aldrich (St. Louis, MO, USA). DMEM-high glucose, A549 lung cancer cells, dimethyl sulfoxide (DMSO), Rocher Cell Proliferation kit (MTT) and trypsin-EDTA solution were purchased from Hyclone (Logan, UT, USA). A human lung adenocarcinoma of alveolar epithelial origin cell line (A549 cells) was bought from Cellonex, Separations (Randburg, South Africa). Amphotericin/Streptomycin/Penicillin (AB/S/P) solution was purchased from Lonza (Bend, OR,USA). Dichloromethane, toluene, acetone, and diethyl ether were procured from Merck (Pty) Ltd., (Modderfontein, South Africa). The reagents used were of analytical grade.

3.2.2. Synthesis of the mPEG-PLGA Copolymer

The double emulsion (W/O/W) process was used to develop the mPEG-PLGA copolymer. PLGA (2.5 g) and mPEG (0.25 g) were reacted overnight at 110° C with vigorous stirring in toluene with stannous octoate (20 mg) as a reaction catalyst. The resultant product was dissolved in dichloromethane (60 mL), precipitated with diethyl ether, filtered, and subsequently added to robustly agitated water (60 °C, 500 mL). The mPEG-PLGA residue was decanted out of the aqueous component after successful DCM evaporation, and subsequently lyophilised [22].

3.2.3. Morphological characterisation of the mPEG-PLGA Nanoparticles

Morphological characterisation of the PTX-loaded NPs was revealed using SEM. Briefly, the optimised PTX-loaded NPs were suspended in ethanol and were mounted directly onto the SEM sample stub, the sample was coated with palladium gold. A field emission scanning electron microscope from Sigma, model 03-39 (Zeiss Jena, Germany), was used to ascertain the surface and shape characterisation of the mPEG-PLGA NPs sample. The characterisation was undertaken at 5 - 15kV acceleration voltage under an argon atmosphere.

3.2.4. Determination of chemical integrity and stability of the mPEG-PLGA Copolymer

A spectrometer by PerkinElmer (Waltham, MA, USA), featuring a single reflection diamond MIRTGS detector was used to acquire the FTIR spectra of the synthesized mPEG-PLGA copolymer, to confirm its purity and chemical structure stability. Pristine PLGA, pristine mPEG and synthesised mPEG-PLGA, were processed by a universal attenuated total reflectance (ATR) polarisation accessory and analysed with a constant pressure of 110 psi at a resolution of 4 cm^{-1} [22]. The ^{1}H NMR spectra of the synthesized mPEG-PLGA copolymeric platform was evaluated using a Bruker AVANCE II 500 MHz equipment (Bruker Avance Biospin, Germany). Deuterated dimethyl sulfoxide was used as the solvent of choice for analysing the samples at 25 °C, and chemical changes were measured. Functional groups were analysed. This technique was used to confirm the crosslinking occurrence, thereby confirming successful organic synthesis of the mPEG-PLGA copolymeric platform.

3.2.5. Preparation of the PTX-loaded mPEG-PLGA nanoparticles and determination of the Drug loading and Encapsulation Efficiency

A single emulsion (O/W) approach was used to synthesize the PTX-loaded mPEG-PLGA nanoparticles (NPs). In a methylene chloride solution (1 mL), mPEG-PLGA (100 mg) and PTX (10 mg) were dissolved. The solution was sonicated in PVA (1 %, 10 mL) aqueous solution after being agitated for 10 minutes under ambient conditions. DCM was evaporated and the resultant solution as centrifuged at 5000 rpm for 20 minutes under ambient conditions, washed twice with distilled water, to obtain the PTX loaded NPs. The amount of PTX loaded into the NPs was calculated using an indirect method that was used to determine the drug content in the NPs by measuring the PTX that was not encapsulated, the calculations were done using Equations 3.1 and 3.2 [23]. The resultant NP solution was centrifuged for 15 minutes at 1000 rpm. The supernatant was then collected and analysed using an ultraviolet-visible (UV) spectrometer at 229.9 nm. A 2-factor Box-Behnken experimental design was generated using Minitab® statistical software (Minitab® Inc., PA, USA), to investigate the effect of varying PVA concentration (1% - 5%). The entrapment efficacy (EE) and drug loading (DL) can be calculated using the following equations:

$$DL\ (\%) = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of nanoparticles}} \times 100 \qquad \text{(Equation 3.1)}$$

$$EE\ (\%) = \frac{\text{Weight of drug in nanoparticles}}{\text{Theoretical drug loading}} \times 100 \qquad \text{(Equation 3.2)}$$

3.2.6. Transferrin (TF) and Fluorescein Isothiocynate (FITC) conjugation onto the PTX-loaded mPEG-PLGA Nanoparticles

Firstly, transferrin (TF) was conjugated onto the PTX-loaded mPEG-PLGA CPNPs. Briefly, PTX-loaded CPNPs (40 mg) were resuspended in 10 mL sodium chloride solution. To this dispersion, 2 mg/ mL of EDC and 2 mg/ mL of NHS were added dropwise. The mixture was stirred for 15 min at room temperature. Then TF (8 mg) was dissolved in phosphate buffered saline (5 mL). the TF solution was then added to the stirring mixture, and left gently stirring for 6 hr. The final mixture was centrifuged to remove excess unreacted reagents, and washed twice, then lyophilised [24].

Subsequently, FITC (1 mg) was conjugated to the TF-PTX-NPs (15 mg) in 200 µL of DMSO stirred in darkness for 15 min. The prepared solution was added dropwise to 4.8 mL of deionised water and stirred for 2h. After 2h, the reaction solution was dialysed against distilled water in a dialysis bag (MWCO 3500). The final mixture was centrifuged to obtain the TF-PTX-FITC-NPs, washed twice, then lyophilised [22].

3.2.7. Particle size analysis of the crosslinked mPEG-PLGA copolymer

A Zetasizer NanoZS series (Malvern Instruments Ltd, Worcestershire, UK) fitted with dynamic light scattering and photon correlation spectroscopy at a fixed scattering of 90° was used to measure the size, polydispersity index, and surface charge (mV) of the mPEG-PLGA copolymeric NPs synthesized. Distilled water (5 mL) was used to solubilise the samples (1 mg),the samples were sonicated to ensure complete dissolution and measurements were obtained at 25°C.

3.2.8. Determination of Crystallinity of the mPEG-PLGA Nanoparticles

The pristine polymers i.e. mPEG and PLGA as well as the lyophilised PTX-loaded mPEG-PLGA copolymer samples were transferred to a container for sample analysis on a benchtop MiniFlex 600 powder X-ray diffractometer (Rigaku, Tokyo, Japan). Data was collected utilizing a 2theta scan at range of 5 – 60°, at a 15° min $^{-1}$ scan rate and using Cu K α radiation at 40 kV and 15 mA. The X-ray diffractometry (XRD) measurement show the polymers and nanoparticles' their amorphous nature and degree of crystallinity, as well as their surface properties and behaviour.

3.2.9. Thermogravimetric Analysis of the mPEG-PLGA Nanoparticles

The physical state of the PTX-free mPEG-PLGA, PTX loaded mPEG-PLGA, as well as the pristine PLGA and mPEG, were evaluated using Differential Scanning Calorimetry. A DSC-1 1100LF calorimeter (Mettler Toledo, Switzerland) was used to investigate the thermal behaviour of mPEG-PLGA NPs, mPEG, and PLGA. Adequate quantities of samples were sealed in aluminium crucibles, and a 10 °C/min heating rate from 0 to 400 °C was applied in a nitrogen environment. Thermogravimetric analysis was carried out under nitrogen environment at a 10 °C/ min rate from 30 to 900 °C. Enthalpy change analysis was calculated using Equation 3.4:

$$\text{Enthalpy change (J/ g)} = \frac{\text{Area under the curve}}{\text{Mass of sample}} \qquad \text{(Equation 3.3)}$$

3.2.10. Determination of the *In Vitro* Cumulative Release of Paclitaxel from the mPEG-PLGA Nanoparticles

A UV spectrophotometer was used to generate a linear calibration curve (Equation 3.5) to assess the *in vitro* release of the model drug, PTX, from the mPEG-PLGA CPNPs (20 mg) in phosphate-buffered saline (5 mL) at pH 1.2, 6.8, and 7.4 using a prepped dialysis tubing (MWCO 3500 Da) immersed in 100 mL of relevant PBS solution. An orbital shaking incubator (LM-530-2, MRC Laboratory Instruments Ltd., Hadistradrut, Holon, Israel) was utilised to provide constant agitation at 37° C for 24 hours. The environmental buffer solution was replaced with fresh phosphate-buffered saline at regular timed intervals, and the concentration of released PTX in the withdrawn phosphate-buffered saline was determined using the previously constructed calibration curve where PTX showed its peak absorbance (229.9 nm), which was quantified using a Lambda 950 Visible-UV spectrophotometer (Perkin Elmer Fremont CA, USA). Equation 3.5 was used to calculate the amount of PTX released over time. Thereafter, the cumulative percentage release of PTX was calculated as a time-dependent variable.

Calibration curve: $y = 0.0448x + 0.0311$ $(R^2 = 0.9999)$ (Equation 3.4)

3.2.11. Evaluation of the *in vitro* cytotoxicity activity of PTX-loaded mPEG-PLGA NPs on A549 cells

A MTT assay was carried out on A549 cells: a cell line from a human lung adenocarcinoma of alveolar epithelial origin, the cells were procured from Cellonex, Separations (Randburg,

South Africa). T75 culture flasks were used to grow the cells at 37° C humidified air, 5 % Carbon dioxide and in DMEM media. The cells were seeded into two 96-well plates at a density of 30 000 cells/ml (90 µl cell suspension per well) and incubated for 24 h. After 24 h, the wells were treated with 10 µl of PTX solution, PTX-loaded mPEG-PLGA, and mPEG-PLGA at different final concentrations of 1.75, 3.5, 7, 14 and 28 µg/ml in triplicates. Dimethyl sulfoxide (DMSO) was used as the solvent of choice, to dissolve the treatment formulations. The DMSO concentration was reduced by serial dilution to 0.1 %. After 24 h and 48 h of incubation, respectively, 10 µl of MTT reagent (Cell proliferation Kit, Roche Diagnostics GmbH, Mannhein, Germany) was added to the respective wells. The MTT solubilising agent was then added to the wells after 4 hrs, and the plate was incubated overnight. A Multilabel plate reader (VICTOR X3, PerkinElmer, USA) was used to measure the cell viability absorbance at 570 nm. Equation 3.6 was employed to ascertain the percentage number of viable cells that were viable.

$$\text{Cell viability (\%)} = \frac{\text{sample} - \text{blank}}{\text{control - blank}} \times 100 \qquad \text{(Equation 3.5)}$$

Statistical significance was computed using GraphPad, the p values ($p < 0.05$) were determined using t-test, at 95% confidence level.

3.2.12. Cellular uptake study

A549 cells were seeded at a 30 000 cells/mL density onto a borosilicate chambered cover glass and incubated for 24 hours to assess cellular uptake of the TF-PTX-FITC-NPs and PTX-FTIC-NPs. Thereafter, 2 mL TF-PTX-FITC-NPs (14 µg/mL) and PTX-FTIC-NPs (14 µg/mL) were introduced into the wells to replace the spent growing media. After treatment, the cells were rinsed three times with phosphate-buffered saline to wash off any remaining treatment solution before being fixed with 4% paraformaldehyde. The fixed cells were then rinsed with PBS to remove any fixation agent. 2 mL of 1 µM CellTracker Deep Red dye (ThermoFisher, South Africa) was added into the wells, which were then incubated for 45 min under 37° C humidified air, 5 % Carbon dioxide conditions. The CellTracker was removed, and the cells washed in triplicate in PBS. In the wells, 2 mL of 300 nM DAPI stain (ThermoFisher, South Africa) was added, and the plate was incubated at room conditions for 5 minutes. The cells were rinsed thrice with PBS after the solution was aspirated. After that, the coverslips were mounted and examined under a fluorescence microscope at a magnification of x40 [24] [25] [26].

3.3. Results and Discussion

3.3.1. Assessment of the Particle Size and Surface Morphology of the mPEG-PLGA Nanoparticles

To ensure extended systemic circulation, the mPEG-PLGA NPs should be small enough (20 nm – 200 nm) to prevent detection and destruction by the reticuloendothelial system [27]. The size of the NPs is crucial in drug cellular uptake, NPs with size <200 nm have been reported to have an improved enhanced permeability and retention effect (EPR) to tumour tissues [28]. A 2-factor Box-Behnken experimental design was generated using Minitab® statistical software (Minitab® Inc., PA, USA), to investigate the effect of varying PVA concentration (1% - 5%) and varying PLGA (50 mg – 300mg). Nine formulations we prepared with varying amounts of PLGA and PVA (Table 3.1), while the mPEG was kept at a constant (100 mg). The size of the PTX loaded nanoparticles was found in the range of 195 – 238 nm. On average, the NP size increased with increase in PLGA (mg). This is most likely owing to the PLGA solution's increased viscosity in organic solution, which results in poor PLGA solution dispersibility into the aqueous phase [29]. All formulations showed uniform particle size distribution, with a PDI between 0.06 and 0.20. As shown in table 3.1, the effect of the PVA concentration was also investigated and there was a reduction in nanoparticle size when the PVA concentration was escalated from 1 % to 5 % (w/v). A stabiliser is used during the emulsification process to avoid coalescence of globules. Therefore, high concentrations of the stabiliser lead to a reduced size of the nanoparticles [29]. The zeta potential values varied between -24 mV and -32 mV. The zeta potential is an important particle characteristic as it is commonly an index of nanoparticle stability.

Table 3.1: Particle size and potential of paclitaxel loaded nanoparticle formulations containing various amounts of PLGA in organic phase and PVA in aqueous phase

PVA (%)	**PLGA (mg)**	**Size (nm)**	**PDI**	**Zeta potential (mV)**
1	50	197.8	0.066	-31.9
	175	234.5	0.123	-31.2
	300	214.3	0.040	-29.0
3	50	217.9	0.096	-25.1
	175	212.8	0.051	-23.7
	300	231.8	0.083	-28.8

5	50	195.7	0.080	-24.1
	175	201.8	0.068	-30.0
	300	200.8	0.053	-27.3

For the optimised formulation, the mPEG-PLGA NPs and PTX laden mPEG-PLGA exhibited particle sizes of 177 nm and 210 nm, respectively, and a polydispersity index of 0.258 and 0.249, respectively. The mPEG-PLGA copolymer had a zeta potential of -31.3 mV. SEM micrograph (Fig. 3.6) proved a spherical shape of the mPEG-PLGA CPNP, with smooth surfaces. The smooth surface of the NPs suggests that the organic solvents were completely removed throughout the synthesis process [30]. The nanoparticles' physical state can impact the release characteristics of therapeutic payload, thereby its curative outcome [31].

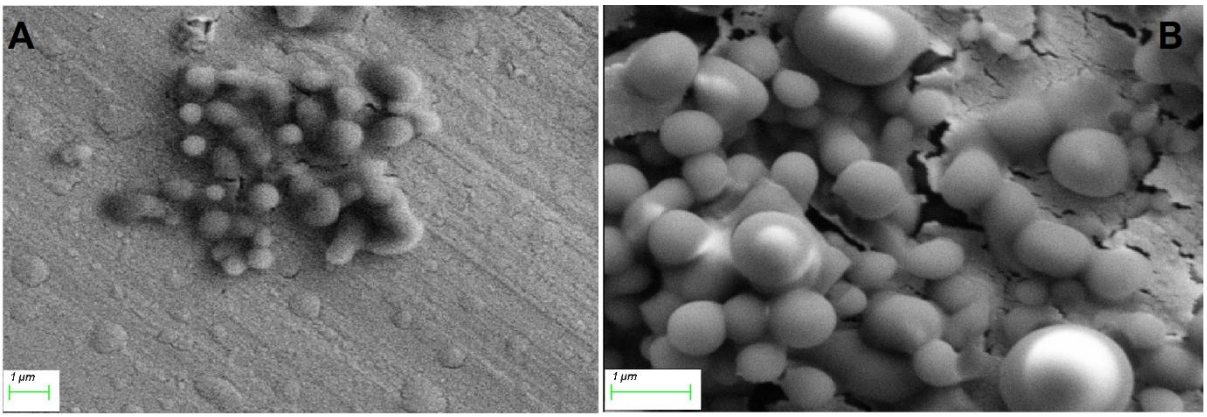


Figure 3.2: Scanning electron micrographs of nanoparticles synthesised using the emulsion solvent evaporation method (**A**) lower magnification (20 K X), (**B**) higher magnification (39.31 K X).

3.3.2. Spectral analysis of the crosslinked mPEG-PLGA copolymer

The FTIR spectra of the pristine polymers i.e. mPEG and PLGA as well as the mPEG-PLGA copolymer are shown in Figure 3.2, and the spectra validated the mPEG-PLGA copolymer's construct. The absorption peak at 3466.76 cm^{-1} was attributed to terminal hydroxyl groups of the mPEG-PLGA. The bands 2923.42 cm^{-1} and 2971.77 cm^{-1} corresponded to the C – H and CH_2 stretches, and the peak at 2871.77 cm^{-1} was attributed to a C – H bonds at –CH-, and the prominent peak observed at 1736.07 cm^{-1}, where crosslinking occurred, was attributed to C=O stretch. The 1119.49 - 1052.95 cm^{-1} peak was linked to the C – O stretch. The PLGA spectra was confirmed by the presence of C – H bonds at 500 - 1550 cm^{-1} , C=O stretch at

1775 cm^{-1}, and the stretching of C – H, CH_2, and CH_3 between 2885 cm^{-1} to 3000 cm^{-1}. The mPEG spectra exhibited a wide OH stretch at 3250 – 3500 cm^{-1}. There is a strong peak at 2800 cm^{-1} due to the C – H stretch, and the complex peak at 1125 cm^{-1} was due to the C- O – C stretch vibrations. In ^{1}H MNR of mPEG-PLGA (figure 2B), the large peak between δ 3.0 ppm and δ 3.5 ppm was attributed to methylene protons of the mPEG, while the combined signals around δ 1.50 ppm were allocated to the $_{D-}$ and $_{L-}$Lactic acid repeat units. Although the considerable complexity of the signals can arise from numerous D-lactic, L-lactic, and glycolic acid sequences in the mPEG-PLGA copolymer backbone, the successful synthesis mPEG-PLGA copolymer was confirmed by characteristic peaks of lactic acid -CH at δ 5.2 ppm and glycolic acid $-CH_2$ protons at δ 4.8 ppm.

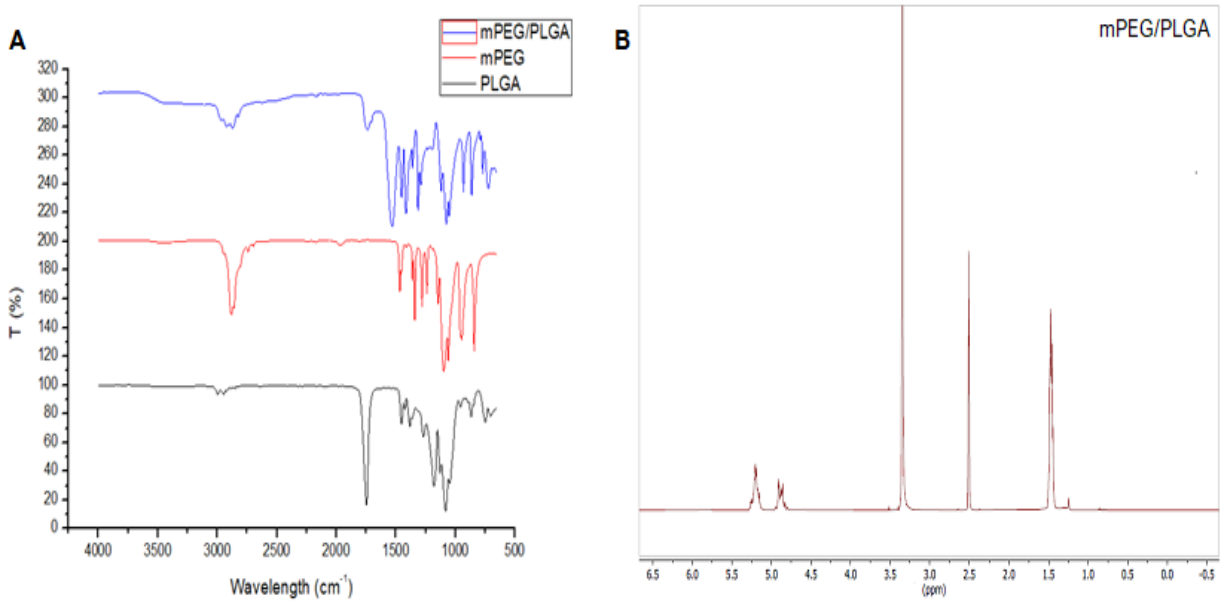


Figure 3.3: Profiles showing a) FTIR spectra of the mPEG-PLGA CPNPs prepared by the solvent evaporation technique, pristine mPEG and pristine PLGA, and b) ^{1}H NMR spectra of the mPEG-PLGA copolymer.

3.3.3. Crystallinity Assessment of the Pristine Polymer and crosslinked mPEG-PLGA copolymer.

XRD is used to ascertain crystallinity of the mPEG-PLGA NPs (Fig. 3.3), the characteristics are crucial for interactions within the circulatory system [31]. The mPEG displayed crystalline peaks at 2θ between 19° and 30°. While an amorphous scattering XRD pattern appears at 2θ from 12° to 25° for the PLGA polymer. The mPEG-PLGA copolymer exhibits an amorphous pattern with slight crystalline diffraction peaks at 2θ of 15° - 25°, showing that the crystalline mPEG peaks are masked in the copolymer, the crystallinity of mPEG decreased in the mPEG-

PLGA CPNP. The surface properties of the mPEG-PLGA suggest that mPEG shielding should be in effect in the formulation. Because the segments of the mPEG-PLGA copolymer become amorphous with the addition of PLGA, the mPEG-PLGA peaks are no longer sharp and narrow in comparison to the pure mPEG.

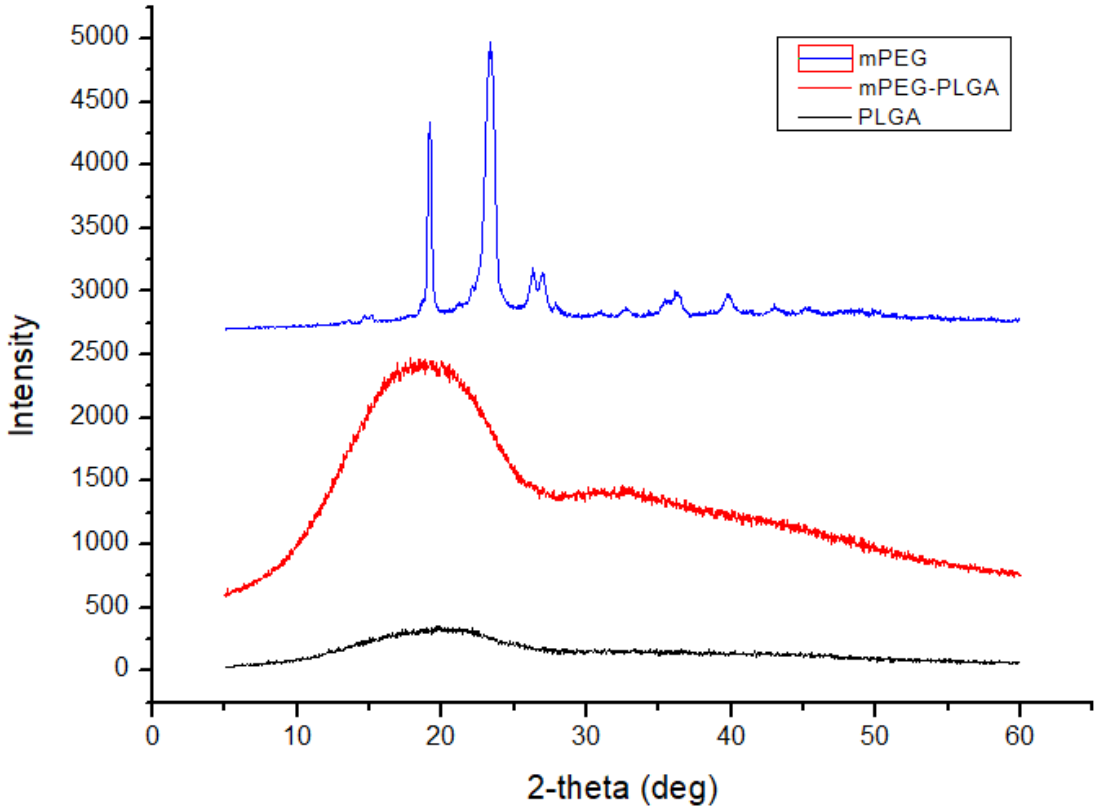


Figure 3.4: XRD Spectra of pristine mPEG, pristine PLGA powder and blank mPEG-PLGA copolymer powder.

3.3.4. Thermal Analysis of PLGA, mPEG and the Blank mPEG-PLGA Nanoparticles

The drug loading and release from the mPEG-PLGA NPs are influenced by their thermal and physical state. TGA results shown in Figure 3.4 reveal that PLGA degradation began around 275°C and the polymer was entirely degraded at around 380°C. mPEG degradation occurred at 400°C and the polymer was completely degraded at 440°C As seen in Figure 3.4 (A) and 3.4 (B), the pristine polymers showed stable thermal degradation. At 255°C, the blank mPEG-PLGA synthesized by the solvent evaporation approach began to degrade, a gradual degradation phase up to 400°C was observed, at which point roughly 40% mPEG-PLGA had

degraded, followed by another phase from 700°C, leaving 50% remnant product at 900°C. A two-step weight loss mechanism is seen in the copolymeric mPEG-PLGA TGA curves. The first process involves the decomposition of carboxylic acid groups, whereas the second involves mPEG scission, after which the loss rate reduces. Because of the coupling of mPEG and PLGA segments, the TGA curve of mPEG-PLGA changed slightly to a lower temperature than that of mPEG and PLGA, indicating that overall stability decreased in the synthesised copolymer as compared to the starting polymers [32].

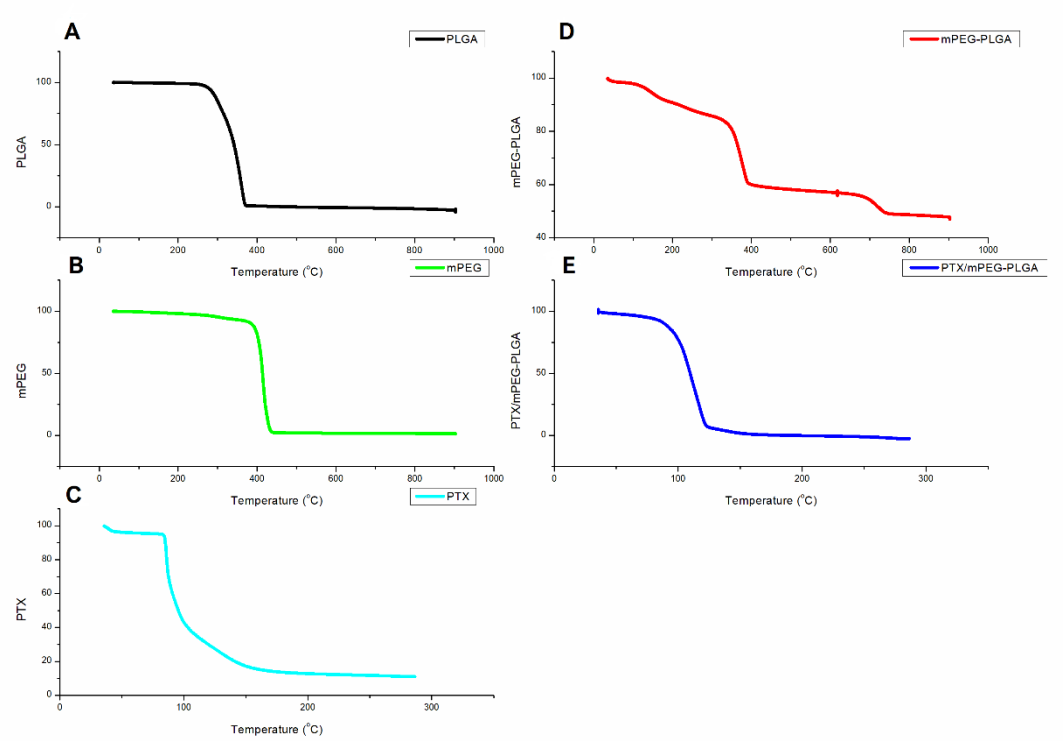


Figure 3.5:Profiles showing thermal degradation of (**A**) pristine PLGA , (**B**) pristine mPEG, (**C**) pristine PTX (**D**) the mPEG-PLGA CPNPs and (**E**) the PTX-loaded mPEG-PLGA CPNPs.

DSC was used to provide the physical status of the pure drug, PLGA, mPEG, blank NPs and drug loaded NPs. The DSC thermographs are shown in Figure 3.5. At 55 °C, the thermograms for blank nanoparticles exhibited a typical endotherm glass transition temperature peak, normally exhibited in PLGA containing NPs for PLGA NPs [33]. PTX exhibits an endothermic peak corresponding to a melting point of 220 °C and exothermic peak at 250 °C, proving its crystalline nature. Furthermore, it can be concluded that paclitaxel was present in a homogenous dispersion within the PLGA matrix. The DSC analysis of the PTX-loaded NPs revealed that no crystalline drug was present in the sample, it suggested that PTX exists in an amorphous state in the NPs.

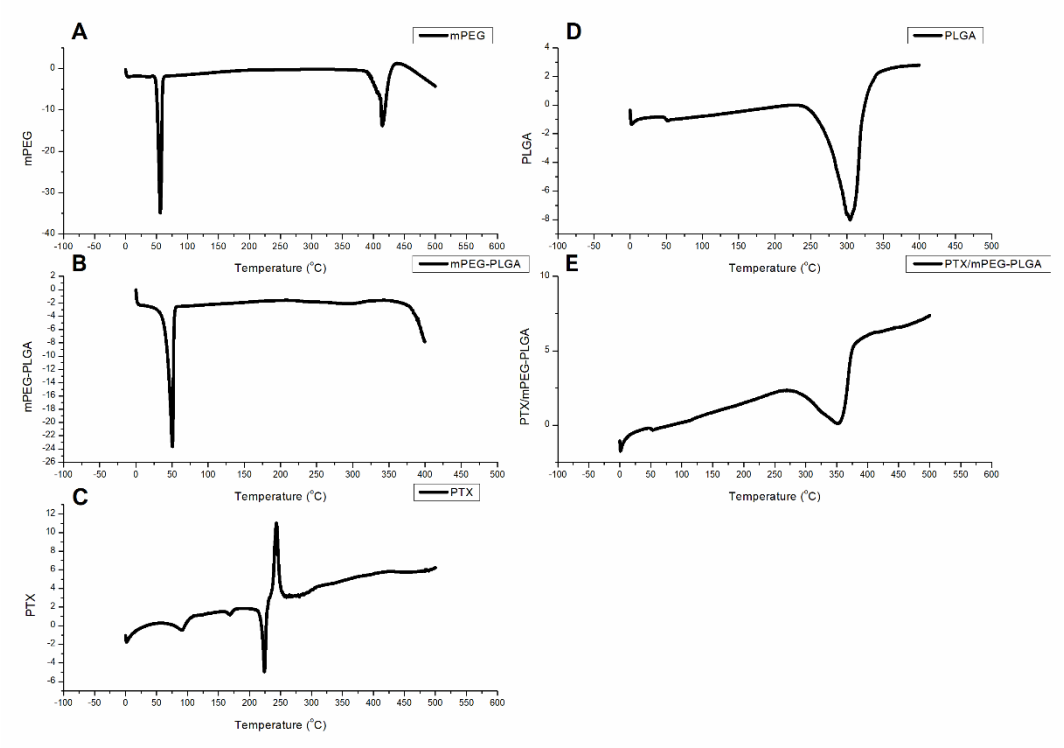


Figure 3.6: Graphs showing thermal degradation profiles of (**A**) methoxy-Poly (ethylene glycol) (mPEG) (**B**) mPEG-PLGA copolymer (**C**) paclitaxel (PTX) (**D**) PLGA (poly (lactic-co-glycolic) acid) (**E**) PTX loaded mPEG-PLGA.

3.3.5. Assessment of the PTX-loading Efficiency and *In Vitro* Drug Release Kinetics

PLGA creates a hydrophobic core and mPEG a hydrophilic corona, hence the mPEG-PLGA copolymer can therefore form a micellar structure [34]. Polymeric drug delivery systems can achieve controlled drug release, which is beneficial for improving the oral bioavailability and reducing the toxicity and side effect of drugs to normal tissues [34].

Table 3.2 shows the EE of PTX in various mPEG-PLGA NP formulation variants designed using the 2-factor Box-Behnken. The nine formulations designed with the variables employed, were loaded with an equal amount of PTX (10 mg). The EE value ranged from 11 - 83 %. The increase in EE was dependent on the quantity of PLGA in the organic phase. PTX entrapment was also directly proportionate to an increase in PLGA and inversely to a decrease in the PVA concentration since higher surfactant concentrations lead to the diffusion of PTX from the inner core to the outer surface [35].

Table 3.2: Results of PTX entrapment and drug loading in the mPEG-PLGA nanoparticles.

PVA Concentration (%)	PLGA (mg)	Entrapment Efficiency (%)	Drug Loading (%)
1	50	34.3	7.6
	175	68.5	6.2
	300	83.9	3.1
3	50	11.3	6.1
	175	28.3	1.1
	300	67.3	2.5
5	50	18.8	4.7
	175	21.2	1.9
	300	52.1	1.7

The ideal formulation exhibited an entrapment efficiency of 78.6% and a drug loading efficiency of 3.74%. The *in vitro* release graphs of PTX-NPs were obtained at pH 1.2, 6.8 and 7.4 (Fig. 3.7). For the initial loading of the PTX onto the mPEG-PLGA NPs, a set amount of drug (10 mg) was employed.

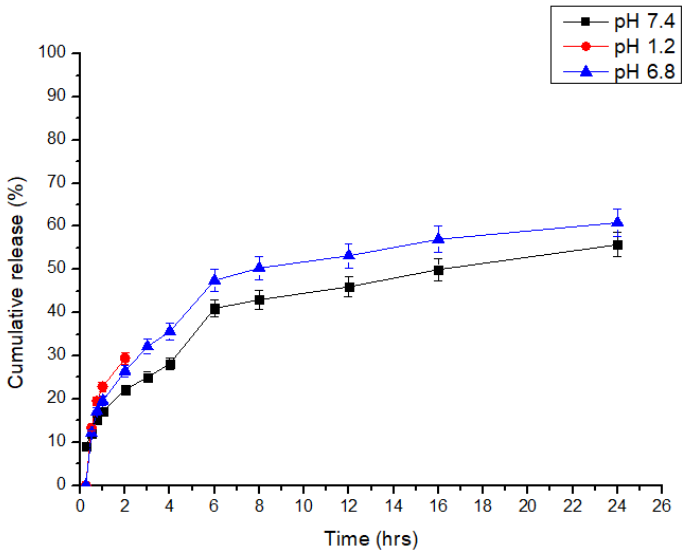


Figure 3.7: Cumulative % release profiles of PTX from the mPEG-PLGA NPs, at pH 1.2 (red), 6.8 (blue) and 7.4 (black).

A burst effect is observed up to 6 hrs. This is associated with the presence of PTX on or nearby the surface of the NPs, due to free PTX adsorbed on the mPEG-PLGA copolymer surface. The burst release is followed by a gradual release of PTX over the remaining period of 24 hrs. PTX release at pH 6.8 and 7.4 showed that 60.85% and 55.73% was released within 24 hrs, respectively. With the residence of PTX in the external surface of the NPs (burst effect) and a portion entrapped within the NP core (plateau), it explains the bimodal PTX release kinetics. A longer time for degradation is required for the release of PTX from the NP core [30] [36]. It can therefore be proposed that the release of PTX from the mPEG-PLGA NPs is controlled by diffusion followed by hydrolytic erosion of the mPEG-PLGA. Drug release evaluated at pH 1.2 showed that after 2 hrs, 29.43% of the drug had been released. Due to the hydrophobic nature of PTX, its release from the PLGA core is also regulated by the external mPEG layer [27].

3.3.6. *In vitro* cytotoxicity of PTX, PTX/ mPEG-PLGA and mPEG-PLGA

The *in vitro* cytotoxicity of PTX, blank crosslinked mPEG-PLGA platform and PTX-loaded CPNPs was evaluated on A549 cells. PTX inhibits the microtubule breakdown and promotes the generation of unstable microtubules, thereby blocking normal microtubule reorganisation which is necessary for mitosis and cell growth [37]. After 24 h and 48 h MTT assay was used to measure cell viability was measured after using. Figure 3.8 shows that the blank NPs did not exhibit significant cytotoxicity against the cell line, even at the highest concentration (28µg/ml) used ($p = 0.9465$). However, the PTX solution and PTX-loaded NPs showed cytotoxicity against the A549 cell line. As observed in Figure 7, 5-FU (10 µg/ml) exhibited 80.5% (24 h) and 64.9% (48 h) cell viability. Negative control cells were treated with 0.1 % DMSO, showed a % cell viability of 99.7%, indicating that the solvent was non-cytotoxic.

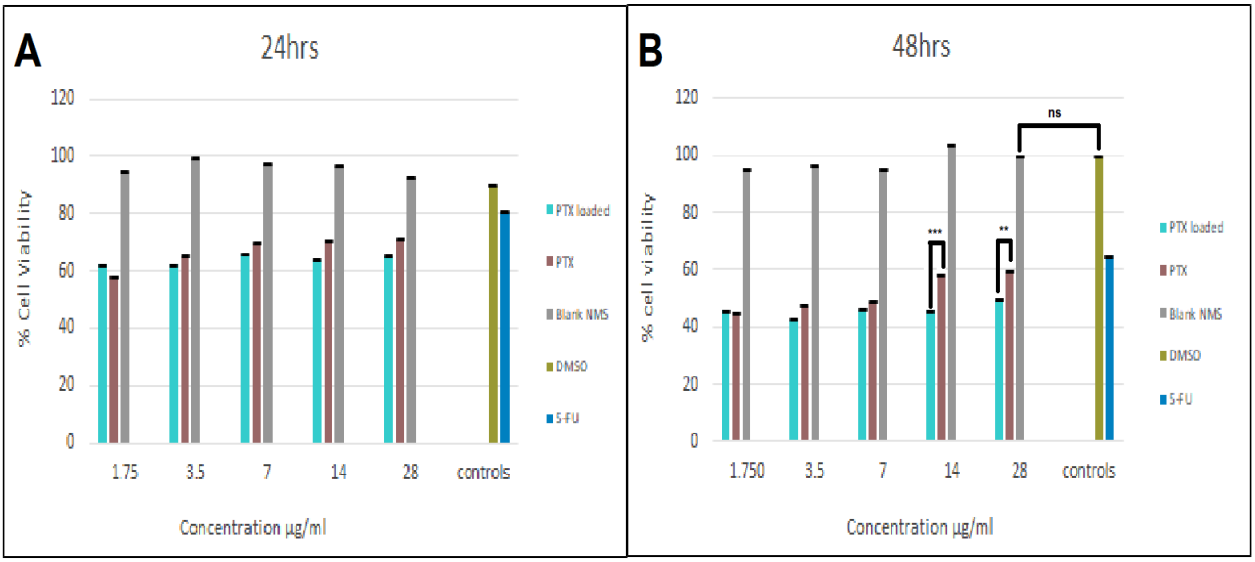


Figure 3.8: Evaluation of cell viability of the A549 cell line that has been treated with paclitaxel (PTX), blank mPEG-PLGA formulation, PTX loaded mPEG-PLGA formulation, dimethyl sulfoxide (DMSO) (0.1 %) and 5-Fluorouracil (10 µg /ml), after 24 h and 48 h. Some error bars fall within the graphs and are not observable. ns: non-significant difference ($p = 0.947$), **** statistically significant difference ($p = 0.0012$) and ***** very statistically significant difference ($p = 0.0001$).

The cells in the untreated wells continued to grow and exhibited significant viability, as shown in Figure 3.8 (A)(B). mPEG and PLGA are non-toxic and biocompatible therefore the NPs synthesised were expected to exhibit those characteristics [38]. This is shown in Figure 3.7 and Figure 3.8(C)(D), at the highest concentration of 28 µg/ml of the blank mPEG-PLGA copolymer, 99.5 % cell viability was observed. The PTX solution showed considerable cell toxicity against the cells as seen in Figure 3.8(E)(F), as also shown by the cell viability of 71.2 % (24 h) - 59.4 % (48 h) at the highest concentration of 28 µg/ml. When compared to the free PTX solution after 48 h of treatment, the PTX-loaded onto the NPs exhibited significantly superior toxicity against the A549 cell line, at 14 µg/ml ($p = 0.0001$) and at 28 µg/ml ($p = 0.0012$). At 28 µg/ml the PTX-loaded mPEG-PLGA showed cell viability of 64.9 % (24 h) and 49.5% (48 h), this is also made evident in Figure 3.8(G)(H). Because of their increased permeability and increased retention impact, mPEG-PLGA NPs can transport the encapsulated PTX to the tumour site [39]. PLGA containing nanoparticles have previously shown to enhance drug cellular uptake, internalisation and biodistribution [40]. Furthermore, the addition of mPEG increases the hydrophilicity of the formulation resulting in a stealth particle with enhanced PTX circulation time and improved pharmacokinetics by avoiding opsonisation [41] [42].

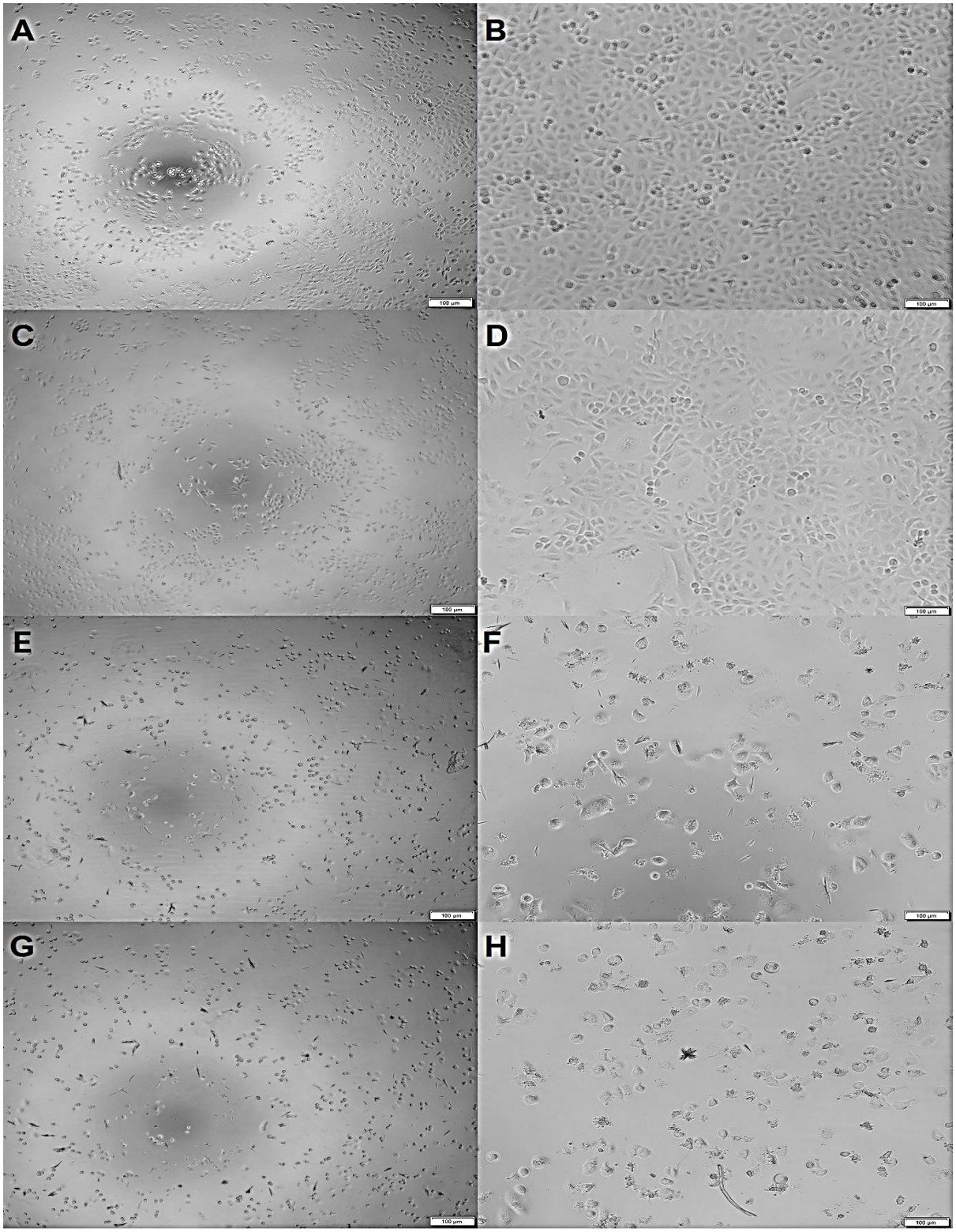


Figure 3.9: Light microscopy images (X10, Olympus CKX53, Tokyo, Japan) of untreated cells after, 24 h (**A**) and 48 h(**B**), cells treated with the blank nanoparticles, after 24 h (**C**) and 48 h (**D**), cells treated with paclitaxel, after 24 h (**E**) and 48 h (**F**), cells treated with the PTX-loaded NPs, after 24 h (**G**) and 48 h (**H**).

3.3.7. Cellular uptake of the FITC labelled PTX-mPEG-PLGA Nanoparticles and FITC labelled TF-PTX-mPEG-PLGA

To investigate the targeted delivery of the nano system, TF-PTX-FITC-NPs and PTX-FTIC-NPs was assessed for preferential cellular uptake by detecting green fluorescence of FITC labelled TF-PTX-NPs and the FITC labelled PTX-NPs internalized into the cells. The red fluorescence reflects the cellular cytosol, and the blue fluorescence reflects cellular nuclei. Figure 3.10 shows the fluorescent images obtained at x40 magnification. The untreated cells maintained cell integrity, with the nuclei and cell outline being clearly visible. After 24 h of incubation the FITC labelled TF-PTX-NPs specific clustered green fluorescence mostly on the cell border, this shows that the TF-PTX-NPs are targeting, compared to the PTX-NPs which showed non-specific green fluorescence covering the entire cell. Endocytosis mediated by Clathrin-dependent receptors could be a fundamental mechanism for TF-PTX-NP absorption in to the A549 cells [43] [44] [45], even although non-specific endocytosis, such as fluid phase macro-pinocytosis, cannot be ruled out. The selective clustered uptake of TF-PTX-NPs by A549 cells verifies the interest in precise targeting, as expected when using an antibody as the targeting moiety [46]. The 24 h incubation allowed for prominent fluorescent intensity, indicating that the nanoparticles were internalised continuously [47]. The treated cells show a notable change in cellular structure due to cell apoptosis, compared to the untreated cell which exhibit normal A549 cell appearance.

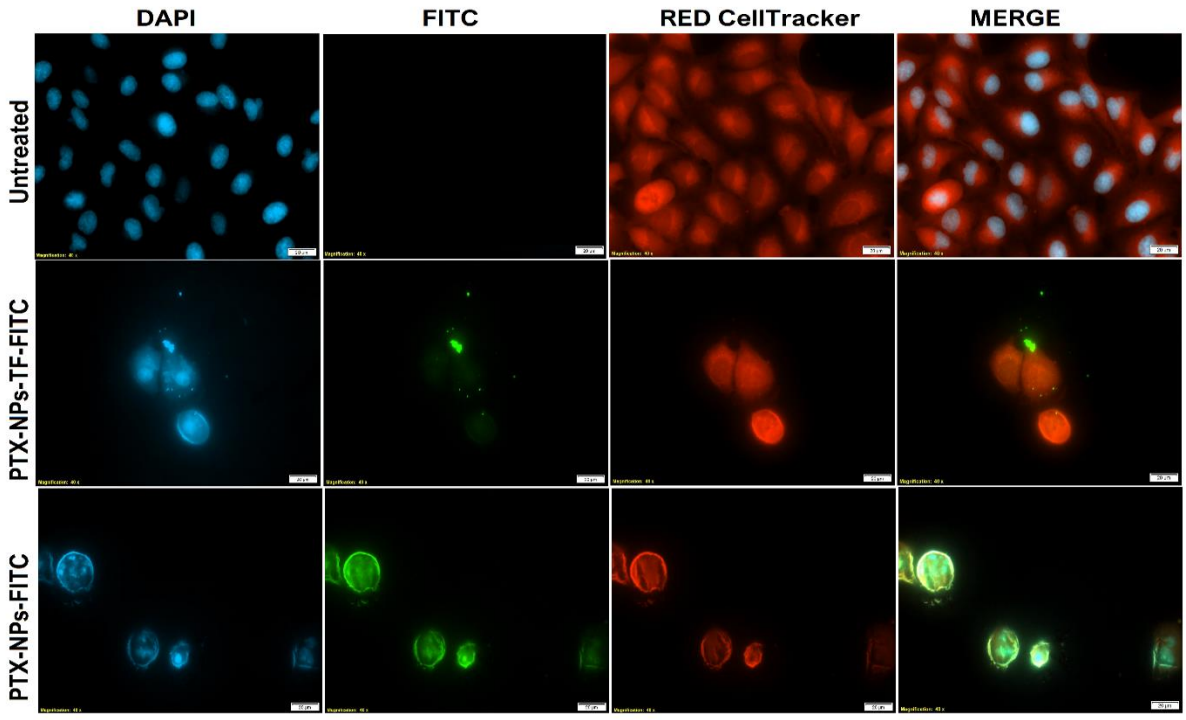


Figure 3.10: Observation of cellular uptake of nanoparticles in A549 cells. The cells were treated with FITC stained TF-PTX-NPs and PTX-NPs for 24 h. The cells were further stained

with CellTracker dep red dye and DAPI stain to obtain the final fluorescent microscope images at x40 magnification.

3.4. Concluding Remarks

The mPEG-PLGA copolymeric NPs were successfully synthesized for the oral delivery of PTX. The average particle size of the optimised crosslinked mPEG-PLGA was 177.2 nm, and exhibited a PDI of 0.258 and a zeta potential was -31.3 mV, which is desirable for oral absorption from the GIT and enhanced oral and neuro-availability of PTX. PTX followed a bi-modal release pattern from the mPEG-PLGA NPs with a burst effect at first, subsequently followed by sustained release over 24 hours. Additionally, the NPs presented favourable chemical integrity, thermal stability and purity when compared to the controls (pristine polymers).

Cytotoxicity studies showed that the crosslinked mPEG-PLGA did not exhibit any significant toxicity to the cell line, it can therefore be concluded that the PTX free mPEG-PLGA is non-toxic. PTX-loaded NPs exhibited significant toxicity to the A549 line, owing to the successful delivery of PTX to the cells. In summary, these results revealed that the PTX-loaded mPEG-PLGA CPNPs may be a capable approach for the oral delivery of PTX.

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CHAPTER FOUR
Conclusion and recommendations

4.1. Conclusions

The study of oral paclitaxel therapeutics encompasses a great deal of innovative research, striving for novel site-specific targeting properties, with enhanced drug solubility and bioavailability, thus displaying significant potential for a new era of oral dosage forms in paclitaxel formulations. The copolymeric mPEG-PLGA platform was developed specifically for the enhanced paclitaxel oral bioavailability and pharmacological activity, with reduced toxicity. Paclitaxel has low aqueous solubility which is one of the major drawbacks making it most difficult to obtain a composite system with complete efficacy for oral delivery.

With reports of severe allergic reactions, aberrant lipoprotein, erythrocyte aggregation, hyperlipidemia, and peripheral neuropathy, the conventional paclitaxel formulation faces various hurdles in terms of its safety profile. Due to the micelle encapsulation of the drug, the cremophor EL alters the pharmacokinetic profile of paclitaxel with a non-linear increase in plasma concentration, limiting paclitaxel absorption in the first hour. As a result, new paclitaxel delivery techniques are of scientific interest in order to avoid the unfavourable toxicities of conventional formulations.

As observed in the results in this thesis, a nano-formulation prepared using mPEG-PLGA was optimised to achieve the desired therapeutically relevant parameters for the oral delivery of paclitaxel. The optimisation studies showed that the mean particle size decreased with decrease in PLGA concentration in the organic phase. All the formulations showed a uniform particle size distribution and there was no substantial difference in surface charges of the nanoparticles which were prepared with different emulsifier concentrations and different polymer ratios. The optimised formulation showed a low particle size, good poly dispersity index and good entrapment efficiency. The paclitaxel loaded formulation was also characterised for the surface, physical and thermal properties, in vitro cytotoxicity using appropriate techniques. The results suggested that the formulation had great potential to be considered as an oral delivery system for paclitaxel. An oral paclitaxel formulation will provide convenient administration and location flexibility for patients and would prevent unnecessary hospitalisation costs. Oral administration of paclitaxel, as opposed to intermittent intravenous exposure, purportedly allows for continuous exposure at low and efficacious doses throughout the treatment duration, providing for a more flexible administration schedule. Continuous low-dose paclitaxel treatment has also been shown to have a greater chemotherapeutic impact by decreasing tumour-associated angiogenesis.

The mPEG-PLGA copolymer had a small favourable size which allows for enhanced permeability retention in the tumour environment, and thus may aid in preferential accumulation of the formulation in the cancer cells. The paclitaxel loaded formulation had 78.6 % entrapment efficiency and 3.74 % drug loading efficiency. A burst release and sustained release of PTX was observed, this may allow for continuous accumulation of the drug and hence low concentrations can be used to reduce potential toxicity. In vitro cytotoxicity tests performed on A4549 cells showed that the mPEG-PLGA was non-toxic and the PTX loaded mPEG-PLGA had higher toxicity than the free paclitaxel in solution. Additionally, the transferrin conjugated nanoparticles presented favourable targeting capability via clathrin-dependent receptor-mediated endocytosis.

In conclusion, the developed mPEG-PLGA copolymer was an effective combination for paclitaxel delivery. The conjugation with transferrin established a promising platform for targeted oral delivery of paclitaxel, to improve therapeutic efficacy at low doses.

4.2. Future Outlook and Recommendations

The process and results of which should also be monitored and assessed using other methods of characterization and analysis, so as to elaborate on the chemical synthesis, and its results, in order to ensure success and enhance reproducibility.

Subsequent, to the success of the developed formulation, as efficacy of the mPEG-PLGA CPNP has already been proven, it is recommended that further studies be undertaken for various chemical modifications, so as to create a standard of functionalities that can be used to alter specific properties such as drug solubility and further improved release profiles.

Paclitaxel highly binds to efflux Protein P-glycoprotein, P-gp, hence another reason for its low oral bioavailability. Paclitaxel's solubility and oral bioavailability have been prioritized in the development of an oral formulation. Oral paclitaxel has been used in combination with an orally available P-gp inhibitor, such as cyclosporine A, to boost systemic exposure.

Thus, considering the above for future research evaluation, we recommend pre-clinical evaluations for *in vivo* analysis of the system regarding serum concentration evaluation which can be designed with robust parameters of optimal drug loading and release kinetics, and considerable effort would be required to eradicate limitations that are minimally presented in this research.

APPENDICES

Review Paper

cancers

MDPI

Review

Nanotechnology-Based Biopolymeric Oral Delivery Platforms for Advanced Cancer Treatment

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Abstract: Routes of drug administration and their corresponding physiochemical characteristics play major roles in drug therapeutic efficiency and biological effects. Each route of delivery has favourable aspects and limitations. The oral route of delivery is the most convenient, widely accepted and safe route. However, the oral route of chemotherapeutics to date have displayed high gastric degradation, low aqueous solubility, poor formulation stability and minimum intestinal absorption. Thus, mainstream anti-cancer drugs in current formulations are not suitable as oral chemotherapeutic formulations. The use of biopolymers such as chitosan, gelatin, hyaluronic acid and polyglutamic acid, for the synthesis of oral delivery platforms, have potential to help overcome problems associated with oral delivery of chemotherapeutics. Biopolymers have favourable stimuli-responsive properties, and thus can be used to improve oral bioavailability of anti-cancer drugs. These biopolymeric formulations can protect gastric-sensitive drugs from pH degradation, target specific binding sites for targeted absorption and consequently control drug release. In this review, the use of various biopolymers as oral drug delivery systems for chemotherapeutics will be discussed.

Keywords: cancer; biopolymers; nanoparticles; targeted drug delivery; cancer nanotechnology; oral delivery

1. Introduction

Majority of global deaths are caused by non-communicable diseases, with cancer ranking as the leading cause of death. In 2015, the World Health Organisation estimated that cancer is one of the leading causes of death in 91 of 172 countries [1]. Lung cancer is the most common type of cancer, with the highest number of new cases in 185 countries followed by breast, prostate and colon cancer, respectively [1]. Cancer-related deaths are projected to increase rapidly, with an estimated number of >20 million by the end of 2025 [2].

Current cancer treatment options include surgery, radiation therapy and/or chemotherapy, or a combination of these [3]. Conventional chemotherapy works by interfering with DNA synthesis in rapidly dividing cancer cells, leading to their death or impeded cell replication. Chemotherapy causes numerous side effects and is known to also affect healthy cells in the body [4]. The inevitable targeting of healthy cells is the major cause of the high mortality rate of cancer patients due to cellular toxicity. Another aggravating factor is the use of high doses of chemotherapeutic agents, since the bioavailability of several cancer drugs is relatively poor [4]. The use of high drug doses leads to elevated toxicity in normal cells, with the risk of drug resistance developing [4]. Most anti-cancer drugs are administered by intravenous infusion (IV) and, in majority of instances, require patients to be hospitalized leading to poor treatment compliance.